

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
21 March 2002 (21.03.2002)

PCT

(10) International Publication Number
WO 02/22685 A2

(51) International Patent Classification⁷: **C07K 16/00**

(21) International Application Number: PCT/US01/28548

(22) International Filing Date:
11 September 2001 (11.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/231,841 11 September 2000 (11.09.2000) US

(71) Applicants and

(72) Inventors: **KUFE, Donald, W.** [US/US]; 179 Grove Street, Wellesley, MA 02181 (US). **OHNO, Tsuneya** [JP/US]; 3-25-8 Nishi-Shinbashi, Minato-ku, Tokyo 105 (JP).

(74) Agent: **MOLONEY, Stephen, J.**; Associate Counsel, Intellectual Property, Ilex Oncology, Inc., 4545 Horizon Hill Blvd., San Antonio, TX 78229 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,

CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

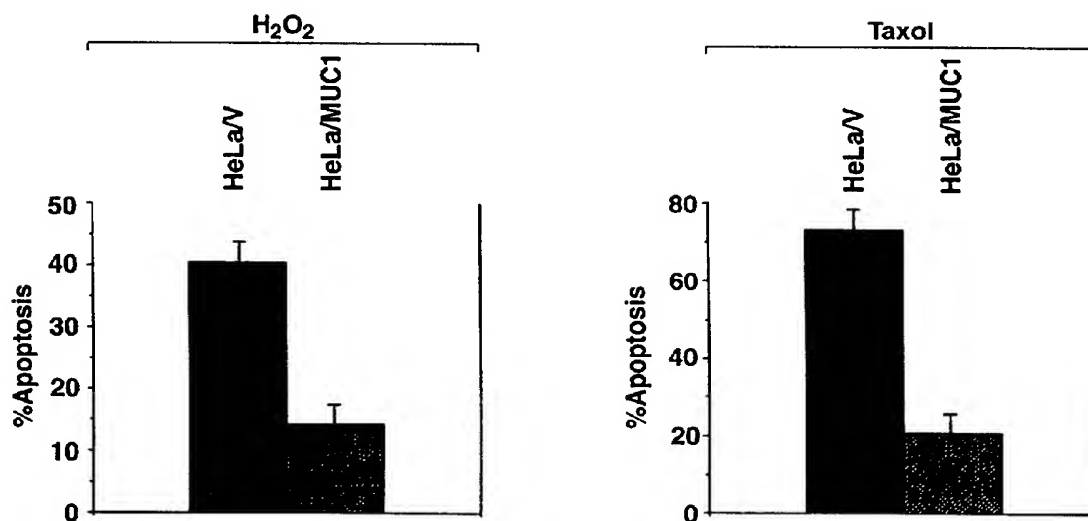
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- without international search report and to be republished upon receipt of that report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MUC1 EXTRACELLULAR DOMAIN AND CANCER TREATMENT COMPOSITIONS AND METHODS DERIVED THEREFROM



(57) Abstract: The present invention provides compositions and methods for inhibiting the proliferation of cancer cells and for the treatment of tumors with antagonists of the binding of ligands to the extracellular domain of MUC1, such binding being related to an oncogenic function of MUC1.



WO 02/22685 A2

MUC1 EXTRACELLULAR DOMAIN AND CANCER TREATMENT COMPOSITIONS AND METHODS DERIVED THEREFROM

This application claims priority to Provisional Application Serial No. 60/231,841,
5 filed September 11, 2000. The United States government may own rights in the present
invention pursuant to grant number R21-CA87421 from the National Cancer Institute,
National Institutes of Health, Department of Health and Human Services.

FIELD OF THE INVENTION

10 The present invention relates generally to the field of cancer therapy and more
specifically to the use of modulators or agents that interact with MUC1 as a point on
intervention in cancer therapy.

BACKGROUND OF THE INVENTION

15 The human MUC1 mucin glycoprotein is expressed on the apical borders of
secretory epithelial cells on the luminal surface of most glandular epithelia (Kufe *et al.*,
1984). In carcinomas, MUC1 is highly overexpressed throughout the entire cell
20 membrane and cytoplasm (Kufe *et al.*, 1984; Perey *et al.*, 1992). As such, the aberrant
pattern of MUC1 expression in carcinoma cells may confer a function for MUC1
normally found at the apical membrane to the entire cell membrane. The hallmark of
MUC1 mucin is an ectodomain comprising a glycosylated 20 amino acid extracellular
sequence that is tandemly repeated 25-100 times in each molecule (Strouss & Decker,
25 1992). The mucin glycosylation level appears to be lower in cancer cells than normal cells
of ductal epithelial tissue (Kufe, U.S. Pat. No. 5,506,343). This hypoglycosylation results
in the exposure of tumor-specific epitopes that are hidden in the fully glycosylated mucin.

Over ninety percent of breast cancers show an increased expression of MUC1
(also known as Mucin, Epithelial Membrane Antigen, Polymorphic Epithelial Mucin,
30 Human Milk Fat Globule Membrane antigen, Episialin, DF-3, etc., *see* Barry & Sharkey,

1985). Several clinical studies have suggested that mucinous tumor antigens expressed on the cell surface of tumor cells associate with poor prognosis of a variety of cancer types (Itzkowitz *et al.*, 1990).

MUC1 is expressed as both a transmembrane form and a secreted form (Finn *et al.*, 1995). The repeating sialyl epitopes of MUC1 (the 'ectodomain') are shed into the serum (Reddish *et al.*, 1996). The N-terminal ectodomain (the extracellular domain that is cleaved) of MUC1 consists of a variable number of the 20-amino acid tandem repeats that are subject to O-glycosylation. This mucin extends far above the cell surface and past the glycocalyx making it easily available for interactions with other cells. The C-terminal region of MUC1 includes a 37 amino acid transmembrane domain and a 72 amino acid cytoplasmic tail that contains sites for tyrosine phosphorylation. A approximately 45-amino acid extracellular domain remains following cleavage of the ectodomain. It is not known what enzyme is responsible for the cleavage of the ectodomain at this time. The extracellular domain or "MUC1/ECD," remaining, after cleavage of the ectodomain, typically includes the amino acid sequence:

TINVHDTVETQFNQYKTEAASRYNLTISDVSVSDVFPFSAQSGAG.

The cytoplasmic domain of MUC1 ("MUC1/CD") encompasses multiple sub-domains that are important in intracellular signaling in cancer cells. β -Catenin binds directly to MUC1/CD at a SAGNGGSSL motif (Yamamoto *et al.*, 1997). β -Catenin, a component of the adherens junctions of mammalian epithelium, binds to cadherins at the intracellular surface of the plasma membrane and performs a signaling role in the cytoplasm as the penultimate downstream mediator of the wnt signaling pathway (Takeichi, 1990; Novak & Dedhar, 1999). The ultimate mediator of the wnt pathway is a nuclear complex of β -catenin and lymphoid enhancer factor/T cell factor (Lef/Tcf) which stimulates the transcription of a variety of target genes (*see e.g.*, Molenaar *et al.*, 1996; Brunner *et al.*, 1997). Defects in the β -catenin-Lef/Tcf pathway are involved in the development of several types of cancers (Novak & Dedhar, 1999).

Glycogen synthase kinase 3 β (GSK3 β) also binds directly to MUC1/CD and phosphorylates serine in a DRSPY site adjacent to the β -catenin binding motif, thereby

decreasing the association between MUC1 and β -catenin (Li *et al.*, 1998). In addition, the c-Src tyrosine kinase also binds to and phosphorylates a MUC1/CD SPYEKV motif, resulting in an increased interaction between MUC1/CD and β -catenin and a decreased interaction between MUC1/CD and GSK3 β (Li *et al.*, 2001).

5 MUC1 associates also constitutively with the epidermal growth factor receptor (EGF-R, HER1) at the cell membrane and activated EGF-R induces phosphorylation of the MUC1/CD SPYKEV motif (Li *et al.*, 2001(a)). EGF-R mediated phosphorylation of MUC1/CD appears to increase the interaction of MUC1 with c-Src and β -catenin and downregulate the interaction between MUC1 and GSK3 β . These results support a model
10 wherein MUC1 integrates the signaling among c-Src, β -catenin and GSK3 β pathways and dysregulation of this integrated signaling by aberrant overexpression of MUC1 in cancer cells could promote the transformed phenotype (Li *et al.*, 2001(a)).

The Armadillo protein p120^{cm} also binds directly to MUC1/CD resulting in the nuclear localization of p120 (Li & Kufe, 2001). P120 has been implicated in cell
15 transformation and altered patterns of p120 expression have been observed in carcinomas (*see e.g.*, Jawhari *et al.*, 1999; Shimazui *et al.*, 1996). P120 is a v-Src tyrosine kinase substrate, binds to E-cadherin, and is implicated as a transcriptional coactivator (Reynolds *et al.*, 1989; Reynolds *et al.*, 1994; Daniels & Reynolds, 1999). The observations that p120 localizes to both cell junctions and the nucleus have supported a role for p120, like
20 β -catenin, in the regulation of both cell adhesion and gene transcription. Decreased cell adhesion resulting from association of MUC1 and p120 may be involved in increased metastatic potential of MUC1-expressing tumor cells.

Thus the available evidence indicates that MUC1/CD functions to transfer signals from the extracellular domain to the nucleus, and utilizes signaling mechanisms that have
25 been implicated in adhesion receptor and growth factor signaling and cellular transformation. It is desirable to identify compositions and methods related to modulation of the MUC1-mediated signaling and its putative role in cellular transformation.

SUMMARY OF THE INVENTION

The present invention encompasses methods of use and pharmaceutical compositions relating to the discovery that the extracellular domain of MUC1 provides
5 binding domains for endogenous ligands and that such binding is related to an oncogenic function of MUC1 and the proliferation of cancer cells.

Broadly the invention relates to cancer treatment compositions and methods employing agents or treatment methodologies that comprise or include antagonists of MUC1/ECD modulated cell proliferation. Preferred are methods and compositions that
10 comprise agents that bind to MUC1/ECD or that bind to MUC1/ECD ligands that activate the oncogenic function of MUC1.

Thus, one aspect of the present invention provides for a method for inhibiting the proliferation of cancer cells, comprising administration of an effective amount of a MUC1/ECD antagonist. MUC1/ECD antagonists are agents that downregulate or reduce
15 the quantity of MUC1/ECD presented on cell surfaces, or downregulate the level of wild-type MUC1/ECD ligands available for binding to MUC1/ECD, and/or MUC1/ECD binding inhibitors. A "MUC1/ECD binding inhibitor" means a compound that inhibits the binding of MUC1 wild-type ligands, which may suitably include neuregulin 2 isoform 5 (SEQ ID NO: 2), neuregulin 2 isoform 6 (SEQ ID NO: 3), and appropriate fragments
20 thereof, to MUC1/ECD or a compound that inhibits the binding of an antibody that binds to an epitope within SEQ ID NO. 4 to MUC1/ECD. Appropriate fragments of neuregulin 2 isoform 5 (SEQ ID NO: 2) and neuregulin 2 isoform 6 (SEQ ID NO: 3) are those that bind to MUC1/ECD. MUC1/ECD binding inhibitors include antibodies, polypeptides and small molecules that inhibit such binding. A "MUC1/ECD-P1 binding inhibitor"
25 means a MUC1/ECD binding inhibitor identified by inhibition of the binding to MUC1/ECD of an antibody the binds to an epitope within SEQ ID NO. 4.

In one embodiment of the invention, the MUC1/ECD inhibitor is the polypeptide of SEQ ID NO: 1, or a fragment comprising at least four consecutive amino acids of SEQ ID. NO: 1 such as TINV, NVHD, VHDV, DVET, VETQ, ETQF, TQFN, QFNQ, FNQY,
30 NQYK, QYKT, YKTE, KTEA, TEAA, EAAS, AASR, ASRY, SRYN, RYNL, YNLT,

NLTI, LTIS, TISD, ISDV, SDVS, DVSV, VSVS, SVSD, VSDV, SDVP, DVPP, VPFP, PFPF, FPFS, PFSA, FSAQ, SAQS, AQSG, QSGA, and SGAG. In other embodiments, the MUC1/ECD inhibitor is a conservative variant of the foregoing peptides. In another embodiment, the MUC1/ECD binding inhibitor is the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, or conservative variants thereof.

In another embodiment of the present invention, the MUC1/ECD inhibitor is an antibody that binds to one or more epitopes in the MUC1/ECD sequence SEQ ID NO: 1. In other embodiments of the invention, the MUC1/ECD inhibitor is an antibody that binds to an epitope within SEQ ID NO: 2 or SEQ ID NO: 3. The antibody may be a polyclonal or a monoclonal antibody. Monoclonal antibodies may be humanized or human monoclonal antibodies. It may also be a bispecific antibody or a fragment which comprises an antigen binding region. In some embodiments, the antibody is conjugated to a chemotherapeutic agent, radioisotope, toxin, or an effector that induces a cytolytic or cytotoxic immune response. Such conjugates may comprise a cytokine, an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic, an alkylating agent, a naturally derived toxin, or an Fc region of a IgG1 immunoglobulin.

In another embodiment, the method further comprises the administration of a chemotherapeutic agent or radiation in combination with a MUC1/ECD antagonist. Chemotherapeutic agents typically include alkylating agents, topoisomerase inhibitors, antimetabolites, tubulin interactive agents, anti-hormonal agents, ornithine decarboxylase inhibitors and tyrosine kinase inhibitors.

In various embodiments, the cancer cells are selected from the group consisting of skin cancer cells, prostate cancer cells, lung cancer cells, brain cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, liver cancer cells, pancreatic cancer cells; colon cancer cells, stomach cancer cells and leukemia cells.

Another aspect of the invention is a method for reducing tumor growth in a mammal comprising administration of a therapeutic amount of a chemotherapeutic agent or radiation and an effective amount of a MUC1/ECD antagonist. In a preferred embodiment, the mammal is human. In one embodiment the method is for treating refractory tumors comprising administration of a therapeutic amount of a

chemotherapeutic agent or radiation and an effective amount of a MUC1/ECD antagonist subsequent to treatment with one or more chemotherapeutic agents. In various embodiments, the tumor is a tumor of the skin, prostate, lung, brain, breast, ovary, cervix, liver, pancreas, colon, stomach or hematopoietic system.

5 Other aspects of the invention relate to pharmaceutical compositions comprising MUC1/ECD antagonists and a pharmaceutically acceptable carrier. Wherein the antagonist is a MUC1/ECD binding inhibitor that may be the polypeptide of SEQ ID NO: 1 or a fragment comprising at least four consecutive amino acids, or conservative variants thereof, and a pharmaceutically acceptable carrier. In some embodiments, the
10 MUC1/ECD inhibitor may be the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, or conservative variants thereof. In other embodiments, the pharmaceutical composition comprises an antibody that is a MUC1/ECD binding inhibitor and binds to an epitope within sequences of the peptides selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 and a pharmaceutically acceptable carrier.

15 The present invention also encompasses methods for screening MUC1/ECD binding inhibitor activity. One embodiment comprises a method of identifying a compound that inhibits the binding of ligands to MUC1/ECD, the method comprising: (a) providing a polypeptide comprising SEQ ID NO: 1 or SEQ ID NO: 5; (b) contacting said polypeptide with a test compound and a ligand to the extracellular domain of MUC1
20 selected from the group consisting of antibodies to MUC1/ECD that stimulate MUC1 mediated cancer cell proliferation and wild type ligands that bind to MUC1/ECD and stimulate cancer cell proliferation; and (c) determining whether the binding of said antibody to MUC1/ECD or wild type ligand is decreased relative to an appropriate control. Appropriate controls include, but are not limited to, assays wherein test
25 compounds are excluded. In one embodiment the MUC1/ECD antibody that stimulates MUC1 mediated cancer cell proliferation is an antibody that binds to an epitope within SEQ ID NO: 4. In other embodiments, the wild type ligands may suitably include neuregulin 2 isoform 5 (SEQ ID NO: 2) and appropriate fragments thereof and neuregulin 2 isoform 6 (SEQ ID NO: 3) and appropriate fragments thereof, wherein appropriate

fragments are those that bind to the SEQ ID NO: 1 and have suitable growth stimulatory activity.

Another embodiment is a method of identifying a compound that inhibits the proliferation of MUC1-expressing cancer cells, the method comprising: (a) providing a
5 population of MUC1-expressing cancer cells; (b) contacting said population of MUC1-expressing cancer cells with a test compound and a ligand to the extracellular domain of MUC1 selected from the group consisting of antibodies to MUC1/ECD that stimulate MUC1 mediated cancer cell proliferation and wild type ligands that bind to MUC1/ECD and stimulate cancer cell proliferation; and (c) determining whether the proliferation of
10 the population of MUC1-expressing cancer cells is decreased by comparison to an appropriate control. Appropriate controls include, but are not limited to, proliferation assays wherein test compounds are excluded. In one embodiment the MUC1/ECD antibody that stimulates MUC1 mediated cancer cell proliferation is an antibody that binds to an epitope within SEQ ID NO. 4. In other embodiments, the wild type ligands
15 may suitable include neuregulin 2 isoform 5 (SEQ ID NO: 2) and appropriate fragments thereof and neuregulin 2 isoform 6 (SEQ ID NO: 3) and appropriate fragments thereof, wherein appropriate fragments are those that bind to the SEQ ID NO: 1 and have suitable growth stimulatory activity.

The present invention also provides methods for identifying compounds that
20 downregulate MUC1/ECD expression. The method comprises: (a) providing a population of MUC1-expressing cancer cells; (b) contacting said population of MUC1-expressing cancer cells with a test compound; (c) utilizing an anti-MUC1/ECD antibody to identify polypeptides comprising MUC1/ECD in the MUC1-expressing cancer cells; and (d) determining whether the expression of polypeptides comprising MUC1/ECD is
25 decreased in comparison to controls wherein the test compound was excluded.

The present invention also encompasses pharmaceutical compositions comprising compounds identified by the foregoing methods and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1: Effect of anti-MUC1-P1 antibody on proliferation of ZR-75-1 breast carcinoma cells.

FIG. 2: Effect of anti-MUC1-P1 antibody on proliferation of SW480 cells stably expressing an empty vector (SW480/V) or MUC1 (SW480/MUC1).

FIG. 3: Effect of ZR-75-1 conditioned medium on proliferation of SW480 cells stably expressing an empty vector (SW480/V) or MUC1 (SW480/MUC1).

FIG. 4: Effect of MUC1 on H₂O₂ and taxol-induced apoptosis in HeLa cells stably expressing an empty vector (HeLa/V) or MUC1 (HeLa/Muc1). The results are expressed as the percentage apoptosis (mean±SE) of three separate experiments.

DETAILED DESCRIPTION OF THE INVENTION

I. Polypeptides

The polypeptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods. Polypeptides can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short polypeptides peptides, such as P0 (SEQ ID NO: 9), PI (SEQ ID NO: 4), P2 (SEQ ID NO: 6) and P3 (SEQ ID NO: 7), by chemical synthesis are well known in the art. Such polypeptides could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, Mass.) or Applied Biosystems-Perkin Elmer (Foster City, CA). Alternatively, segments of such polypeptides could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson *et al.*, (1994). During chemical synthesis of such polypeptides, substitution of any amino acid is achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

Wild-type MUC1/ECD ligand polypeptides can be identified as exemplified in Example 3 herein. Recombinant MUC1/ECD ligands can then be prepared by methods known in the art.

The polypeptides of the present invention include variant polypeptides. By
5 "variant" polypeptide is intended a polypeptide sequence modified by deletion or addition of one or more amino acids at one or more sites in the sequence; or substitution of one or more amino acids at one or more sites within the sequence. Variant polypeptides encompassed by the present invention retain the desired biological activity of the polypeptide from which they are derived. Such variants will have at least 40%, 50%,
10 60%, 70%, generally at least 75%, 80%, 85%, preferably about 90% to 95% or more, and more preferably about 98% or more sequence identity to the amino acid sequence of the polypeptide from which they are derived. The percentage of sequence identity, also termed homology, between a polypeptide native and a variant sequence may be determined by comparing the two sequences using the Gap program (Wisconsin
15 Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), which uses the algorithm of Smith and Waterman, (1981).

The polypeptides of the present invention also include variant polypeptides with one or more conservative substitutions. For the purposes of classifying amino acid
20 substitutions as conservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gin, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions
25 between amino acids in the same class.

Also encompassed by the present invention are chemical derivatives of polypeptides. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized residues include, for example, those molecules in which free amino groups have been
30 derivatized to form amine hydrochlorides, *p*-toluene sulfonyl groups, carbobenzoxy

groups, *t*-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole group of histidine may be derivatized to form N-imbenzylhistidine.

The term "polypeptide" as used herein indicates a molecular chain of amino acids and does not refer to a specific length of the product.

II. Antibodies

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity.

Methods for generating polyclonal antibodies are well known in the art. Briefly, a polyclonal antibody is prepared by immunizing an animal with an antigenic composition and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera including rabbit, mouse, rat, hamster, guinea pig and goat.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, carbodiimide and bis-biotinized benzidine. As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the

immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The serum for an immunized animal may be used as is for various applications or the desired antibody fraction may be purified by well-known methods, such as affinity
5 chromatography using another antibody or a peptide bound to a solid matrix.

Monoclonal antibodies (MAbs) may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified expressed
10 polypeptide. The immunizing composition is administered in a manner that effectively stimulates antibody producing cells.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. The use of rats may provide certain advantages (Goding, 1986), but mice are preferred, with the BALB/c mouse being
15 the most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are
20 preferred. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and obtaining lymphocytes from the spleen.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the
25 animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and have enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of only the desired fused cells (hybridomas). Selected hybridomas are serially diluted and cloned into individual antibody-producing cell lines,
30 which can then be propagated indefinitely to provide MAbs.

In accordance with the present invention, fragments of the monoclonal antibody of the invention can be obtained from the monoclonal antibody produced as described above, by methods which include digestion with enzymes such as pepsin or papain and/or cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated synthesizer, or by expression of full-length gene or of gene fragments in *E. coli* or other recombinant microorganisms and cell lines.

The present invention also encompasses various antibody conjugates. Conjugates with fluorescein markers are prepared by methods known in the art, such as conjugation in the presence of coupling agents or by reaction with an isothiocyanate. Conjugates with metal chelates are similarly produced. Other moieties to which antibodies may be conjugated include radionuclides such as ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , ^{211}At , ^{188}Re , ^{109}Pd , ^{47}Sc , ^{212}Pb , and ^{153}Sm and the like, as described in Gansow, 1991, which is herein incorporated by reference.

Monoclonal antibodies of the invention can also be coupled to conventional chemotherapeutic agents such as an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic or an alkylating agent. Drugs that may be coupled to the antibodies for targeting include compounds such as doxorubicin, cyclophosphamide, cisplatin, adriamycin, estramustine, fluorouracil, ethinyl estradiol, mitoxantrone, methotrexate, finasteride, taxol, and megestrol. Methods of coupling may be direct via covalent bonds, or indirect via linking molecules, and will generally be known in the art for the particular drug selected and are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bisazido compounds such as bis-(R-azidobenzoyl) hexanediamine, bisdiazonium derivatives such as bis-(R-diazoniumbenzoyl)ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. (*See, e.g., Thorpe et al., 1982, herein incorporated by reference*).

The antibodies of the present invention may also be conjugated with various toxin molecules or an effector such as IgG1 immunoglobulin, which induces cytolytic or cytotoxic immune response. Thus, the two components may be chemically bonded together by any of a variety of well-known chemical procedures. For example, the linkage
5 may be by way of heterobifunctional cross-linkers, e.g. SPDP, carbodiimide, glutaraldehyde, or the like. The toxin molecules may also be fused to the antibody or binding regions thereof by recombinant means, such as through the production of single chain antibodies. The genes encoding protein chains may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art (*see e.g.*,
10 Sambrook *et al.*, 1989). The recombinant production of various immunotoxins is well-known within the art and can be found, for example in Thorpe *et al.*, 1982(a), Waldmann, 1991, and Pastan *et al.*, 1992, all herein incorporated by reference. A variety of toxin molecules are suitable for use as the cytotoxic domain in the antibody conjugates or fusion proteins described here. Any toxin known to be useful as the toxic component of
15 an immunotoxin may be used, preferably a protein toxin that may be recombinantly expressed. Particularly useful as the cytotoxic domain are bacterial toxins such as *Pseudomonas* exotoxin A (PE), diphtheria toxin, shiga toxin and shiga-like toxin, and ribosome inactivating toxins derived from plants and fungi, including ricin, α -sarcin, restrictotocin, mitogellin, tricanthosin, saporin-G, saporin-1, momordin, gelonin,
20 pokeweed antiviral protein, abrin, modeccin and others described in Genetically Engineered Toxins, ed. A. Frankel, Marcel Dekker, Inc., 1992, herein incorporated by reference, and any recombinant derivatives of those proteins (*see* Olsnes 1981; U.S. Pat. No. 4,675,382; and U.S. Pat. No. 4,894,443, herein incorporated by reference).

The antibody may also be a bispecific antibody which recognizes both the
25 MUC1/ECD and an antigen which promotes the release of a cytokine such as IL-1, TNF alpha and CD16, CD2, CD3 L.C. CD28, which in turn, will activate the release of IFN gamma or TNF alpha, respectively.

The MAb's of the present invention encompass chimeric Mabs, including, "humanized" forms of non-human (e.g., murine) Mabs. Humanized MAb's are chimeric
30 antibodies which contain minimal sequence derived from non-human immunoglobulin.

For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. (see Jones *et al.*, 1986; Riechmann *et al.*, 1988; and Presta, 1992). Fully human MAbs are preferred in the therapeutic methods of the present invention.

"Single-chain FV" or "sFv" antibody fragments of the present invention comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding (see Pluckthun, 1994).

III. Screening and Diagnostic Assays

The present invention provides for methods for identifying compounds that inhibit the binding of various ligands to MUC1/ECD. The binding ligands include neuregulin 2 isoform 5 (SEQ ID NO: 2), neuregulin 2 isoform 6 (SEQ ID NO: 3) and fragments of either isoform that bind to MUC1/ECD and, in a preferred embodiment, an antibody that binds to an epitope within SEQ ID NO: 4.

In one embodiment, the screening method utilizes an *in vitro* competitive binding assay, wherein the capacity of a test compound to inhibit the binding of the

aforementioned ligands to a polypeptide comprising SEQ ID NO.1 or SEQ ID NO: 5 is assessed. In such an assay, the polypeptide comprising MUC1/ECD derived sequences SEQ ID NO.1 or SEQ ID NO: 5 may be conjugated to another protein or produced as a fusion protein, *e.g.*, the GST-MUC1/ECD fusion protein exemplified herein in Example 3. Other suitable conjugates and fusion proteins may be made by one of skill in the art utilizing procedures known in the art. The polypeptides or MUC1/ECD ligands may be labeled with a radioisotope or fluorescent label (*e.g.*, phycobiliproteins, such as phycoerythrin and allophycocyanins, fluorescein and Texas red). Alternatively an enzyme, such as peroxidase, may be used and conjugated either directly or indirectly via a biotin and avidin or streptavidin system. Decreased binding upon introduction of a test compound is indicative of competitive binding.

A compound that inhibits the binding of ligands to MUC1/ECD may be a modulator, that is an antagonist or agonist of the biological activity initiated by MUC1/ECD binding by neregulin 2 isoforms 5 or 6. *E.g.*, the antibody raised to polypeptide P1 (SEQ ID. NO 4) is expected to inhibit binding of the wild-type ligands but acts as an agonist for the MUC1/ECD binding site, *i.e.*, it stimulates proliferation of carcinoma cells. In contrast, appropriate compounds, such as the MUC1/ECD polypeptide SEQ ID NO. 1, will bind to the endogenous wild-type ligands thereby preventing binding to MUC1/ECD and consequently acting as an antagonist, *i.e.*, preventing or decreasing the proliferation of carcinoma cells that would be otherwise observed upon binding of the MUC1/ECD ligands.

An alternative screening assay can discriminate between MUC1/ECD binding inhibitors that exhibit antagonist and agonist activity in regard to the proliferation of MUC1-expressing cancer cells. The method requires a population of MUC1-positive cancer cells, preferably human cancer cells. This could be a population of cells that constitutively expresses MUC1, but the population is preferably of a cell type engineered to express MUC1. The latter are more versatile in regard to providing cells for appropriate controls, *e.g.* cells engineered with an empty vector, and also for enabling the construction of cells expressing MUC1 mutants. Examples of engineered MUC1 cancer cells include, but are not limited to, SW480 and HCT116 colon cancer cells as

exemplified in Examples 2 and 4 herein. Inhibition of MUC1/ECD ligand-induced cell proliferation will indicate a test compound with antagonist activity. Controls may comprise incubation of cancer cells engineered with an empty vector (*i.e.* MUC1-negative) or incubation of MUC1-positive cells in the absence of either the test
5 compound or the MUC1/ECD ligand. One of the latter controls will identify agonists, *i.e.*, stimulation of cancer cell proliferation observed in incubations in which the test compound is present and the MUC1/ECD ligand is absent. Specificity of the agonist activity is established by use of engineered MUC1-negative cells.

Yet another screening assay monitors MUC1/ECD ligand induced
10 phosphorylation of the intracellular domain of MUC1. Alternate screening methodologies employ monitoring of MUC1/ECD ligand induced association of MUC1 with EGF-R, s-Src, β -catenin, GSK3 β or p120. Methods for monitoring such phosphorylation and protein associations are described in Li *et al.*, (1998), Li *et al.*, (2001), Li *et al.*, (2001(a)) and Li & Kufe, (2001), all herein incorporated by reference.

15 The present invention also provides for methods for identifying compounds that downregulate the expression of MUC1/ECD. In some embodiments of the invention, labeled antibodies to MUC1/ECD are utilized to visualize the expression of MUC/ECD in appropriate cell lines by flow cytometry or by immunohistochemistry, using methods know in the art. Alternatively, the expression of MUC1 can be estimated by
20 immunoblotting or by probing total cellular RNA with labeled DNA probes, *e.g.*, as described in Example 7 herein.

Estimation of the expression of MUC1/ECD can also be used for diagnostic methodologies, wherein antibodies to MUC1/ECD are utilized to investigated the expression of MUC1/ECD on or in cells derived from a subject. Such antibodies can also
25 be utilized for imaging of cancer cells within a subject. Imaging is performed by labeling the anti-MUC1/ECD antibody, *e.g.*, with a radiolabel, and injecting the antibody to a subject and monitoring the location of the antibody within the body of said subject.

IV. Combination with Chemotherapeutic Agents

The present invention encompasses the use of the MUC1/ECD antagonists in combination with chemotherapeutic agents. While not being limited by any particular theory, MUC1 inhibits the apoptotic response to genotoxic stress induced by certain chemotherapeutic agents, and thereby induces resistance to such agents. MUC1/ECD antagonists may be used to mitigate this MUC1 mediated response to chemotherapeutic agents, thereby enhancing the effectiveness of such agents. In this regard, MUC1/ECD antagonists will be useful for the treatment cancer cells resistant to chemotherapeutic agents, including residual cancers remaining or reoccurring after cancer chemotherapy. The foregoing rational also pertains to the combination of MUC1/ECD antagonists and ionizing radiation.

The chemotherapeutic agents useful in the methods of the invention include the full spectrum of compositions and compounds which are known to be active in killing and/or inhibiting the growth of cancer cells. The chemotherapeutic agents, grouped by mechanism of action include DNA-interactive agents, antimetabolites, tubulin interactive agents, anti-hormonals, anti-virals, ODC inhibitors and other cytotoxics such as hydroxy-urea. Any of these agents are suitable for use in the methods of the present invention.

DNA-interactive agents include the alkylating agents, *e.g.*, cisplatin, cyclophosphamide, altretamine; the DNA strand-breakage agents, such as bleomycin; the intercalating topoisomerase II inhibitors, *e.g.*, dactinomycin and doxorubicin); the nonintercalating topoisomerase II inhibitors such as, etoposide and teniposide; and the DNA minor groove binder plicamycin.

The alkylating agents form covalent chemical adducts with cellular DNA, RNA and protein molecules and with smaller amino acids, glutathione and similar chemicals. Generally, these alkylating agents react with a nucleophilic atom in a cellular constituent, such as an amino, carboxyl, phosphate, sulfhydryl group in nucleic acids, proteins, amino acids, or glutathione. The mechanism and the role of these alkylating agents in cancer therapy is not well understood. Typical alkylating agents include: nitrogen mustards, such as chlorambucil, cyclophosphamide, ifosfamide, mechlorethamine, melphalan, uracil mustard; aziridine such as thiotepa; methanesulphonate esters such as busulfan;

nitroso ureas, such as carmustine, lomustine, streptozocin; platinum complexes such as cisplatin, carboplatin; bioreductive alkylators, such as mitomycin and procarbazine, dacarbazine and altretamine; DNA strand-breaking agents including bleomycin.

Topoisomerases are ubiquitous cellular enzymes which initiate transient DNA strand breaks during replication to allow for free rotation of the strands. The functionality of these enzymes is critical to the replication process of DNA. Without them, the torsional strain in the DNA helix prohibits free rotation, the DNA strands are unable to separate properly, and the cell eventually dies without dividing. Topo I links to the 3'-terminus of a DNA single strand break, while Topo II links to the 5'-terminus of a double strand DNA break. DNA topoisomerase II inhibitors include the following: intercalators such as amsacrine, dactinomycin, daunorubicin, doxorubicin, idarubicin and mitoxantrone; nonintercalators such as etoposide and teniposide; camtothecins including irinotecan (CPT-II) and topotecan. A representative DNA minor groove binder is plicamycin.

The antimetabolites generally exert cytotoxic activity by interfering with the production of nucleic acids by one or the other of two major mechanisms. Some of the drugs inhibit production of the deoxyribonucleoside triphosphates that are the immediate precursors of DNA synthesis, thus inhibiting DNA replication. Some of the compounds are sufficiently like purines or pyrimidines to be able to substitute for them in the anabolic nucleotide pathways. These analogs can then be substituted into the DNA and RNA instead of their normal counterparts. The antimetabolites useful herein include: folate antagonists such as methotrexate and trimetrexate; pyrimidine antagonists such as fluorouracil, fluorodeoxyuridine, azacitidine, cytarabine, and floxuridine; purine antagonists include mercaptopurine, 6-thioguanine, fludarabine, pentostatin; sugar modified analogs include cytarabine, fludarabine; ribonucleotide reductase inhibitors include hydroxyurea.

Tubulin interactive agents interfere with cell division by binding to specific sites on Tubulin, a protein that polymerizes to form cellular microtubules. Microtubules are critical cell structure units. When the interactive agents bind on the protein, the cell

cannot properly form microtubules. Tubulin interactive agents include vincristine and vinblastine, both alkaloids and the taxanes (paclitaxel and docetaxel).

Although their mechanisms of action are different, both taxanes and vinca alkaloids exert their biological effects on the cell microtubules. Taxanes act to promote the polymerization of tubulin, a protein subunit of spindle microtubules. The end result is the inhibition of depolymerization of the microtubules, which causes the formation of stable and nonfunctional microtubules. This disrupts the dynamic equilibrium within the microtubule system, and arrests the cell cycle in the late G₂ and M phases, which inhibits cell replication.

Like taxanes, vinca alkaloids also act to affect the microtubule system within the cells. In contrast to taxanes, vinca alkaloids bind to tubulin and inhibit or prevent the polymerization of tubulin subunits into microtubules. Vinca alkaloids also induce the depolymerization of microtubules, which inhibits microtubule assembly and mediates cellular metaphase arrest. Vinca alkaloids also exert effects on nucleic acid and protein synthesis; amino acid, cyclic AMP, and glutathione synthesis; cellular respiration; and exert immunosuppressive activity at higher concentrations.

Antihormonal agents exert cytotoxic activity by blocking hormone action at the end-receptor organ. Several different types of neoplasm require hormonal stimulation to propagate cell reproduction. The antihormonal agents, by blocking hormone action, deprive the neoplastic cells of a necessary stimulus to reproduce. As the cells reach the end of their life cycle, they die normally, without dividing and producing additional malignant cells. Antihormonal agents are typically derived from natural sources and include: estrogens, conjugated estrogens and ethinyl estradiol and diethylstilbestrol, chlortrianisen and idenestrol; progestins such as hydroxyprogesterone caproate, medroxyprogesterone, and megestrol; androgens such as testosterone, testosterone propionate; fluoxymesterone, methyltestosterone.

Adrenal corticosteroids are derived from natural adrenal cortisol or hydrocortisone. They are used because of their anti-inflammatory benefits as well as the ability of some to inhibit mitotic divisions and to halt DNA synthesis. these compounds include prednisone, dexamethasone, methylprednisolone, and prednisolone.

Leutinizing-releasing hormone agents or gonadotropin-releasing hormone antagonists are used primarily in the treatment of prostate cancer. These include leuprolide acetate and goserelin acetate. They prevent the biosynthesis of steroids in the testes.

5 Anti-hormonal agents include antiestrogenic agents such as tamoxifen, antiandrogen agents such as flutamide, and antiadrenal agents such as mitotane and aminoglutethimide.

ODC (or ornithine decarboxylase) inhibitors inhibit cancerous and pre-cancerous cell proliferation by depleting or otherwise interfering with the activity of ODC, the rate limiting enzyme of polyamine biosynthesis important to neoplastic cell growth. In particular, polyamine biosynthesis wherein ornithine is converted to the polyamine, putrescine, with putrescine being subsequently by converted to spermidine and spermine appears to be an essential biochemical event in the proliferation of neoplastic growth in a variety of cancers and cancer cell lines and the inhibition of ODC activity or depletion of ODC in such neoplastic cells has been shown to reduce polyamine levels in such cells leading to cell growth arrest; more differentiated cell morphology and even cellular senescence and death. In this regard, ODC or polyamine synthesis inhibitors are considered to be more cytotoxic agents functioning to prevent cancer reoccurrence or the conversion of pre-cancerous cells to cancerous cells than cytotoxic or cell killing agents. A suitable ODC inhibitor is eflornithine or α -difluoromethyl-ornithine, an orally available, irreversible ODC inhibitor, as well as a variety of polyamine analogs which are in various stages of pre-clinical and clinical research.

Other cytotoxics include agents which interfere or block various cellular processes essential for maintenance of cellular functions or cell mitosis as well as agents which promote apoptosis. In this regard, hydroxyurea appears to act via inhibitors of the enzyme ribonucleotide reductase whereas asparaginase enzymatically converts asparagine into non-functional aspartic acid thereby blocking protein synthesis in a tumor.

Compositions of the MUC1/ECD antagonists of present invention can also be used in combination with antibodies to HER-2, such as Trastuzumab (Herceptin (H)). In addition, the present invention also encompasses the use of MUC1 domain antagonists in combination with epidermal growth factor receptor-interactive agents such as tyrosine

kinase inhibitors. Tyrosine kinase inhibitors suitably include imatinib (Norvartis), OSI-774 (OSI Pharmaceuticals), ZD-1839 (AstraZeneca), SU-101 (Sugen) and CP-701 (Cephalon).

When used in the treatment methods of the present invention, it is contemplated that the chemotherapeutic agent of choice can be conveniently used in any formulation which is currently commercially available, and at dosages which fall below or within the approved label usage for single agent use.

V. Ionizing Radiation

In the present invention, the term "ionizing radiation" means radiation comprising particles or photons that have sufficient energy or can produce sufficient energy *via* nuclear interactions to produce ionization (gain or loss of electrons). An exemplary and preferred ionizing radiation is an x-radiation. Means for delivering x-radiation to a target tissue or cell are well known in the art. The amount of ionizing radiation needed in a given cell generally depends on the nature of that cell. Means for determining an effective amount of radiation are well known in the art. Used herein, the term "an effective dose" of ionizing radiation means a dose of ionizing radiation that produces cell damage or death when given in conjunction with the MUC1/ECD antagonists of the present invention, optionally further combined with a chemotherapeutic agent.

Dosage ranges for x-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

Any suitable means for delivering radiation to a tissue may be employed in the present invention, in addition to external means. For example, radiation may be delivered by first providing a radiolabeled antibody that immunoreacts with an antigen of the tumor, followed by delivering an effective amount of the radiolabeled antibody to the tumor. In addition, radioisotopes may be used to deliver ionizing radiation to a tissue or cell.

VI. Downregulation of MUC1/ECD Expression

The present invention also encompass compounds that downregulate MUC1/ECD expression. One such compound is the isocoumarin NM-3 (2-(8-hydroxy-6-methoxy-1-oxo-1 *H*-2-benzopyran-3-yl) propionic acid). NM-3 and other isocoumarins suitable to
5 downregulate the expression of MUC1/ECD are disclosed in U.S. patent No. 6,020,363, the entirety of which is herein incorporated by reference. Other suitable compounds include 2-substituted estradiol compounds such as 2-methoxyestradiol and 2-hydroxyrestradiol. These and other suitable estradiol derivatives are disclosed in U.S. Patent No. 6,239,123, the entirety of which is herein incorporated by reference. Other
10 compounds suitable for downregulating MUC1/ECD expression include antisense oligonucleotides that target nucleic acid molecules encoding MUC1, as described below.

VII. Antisense Oligonucleotides

The present invention also employs antisense compounds, particularly
15 oligonucleotides, for use in modulating the function of nucleic acid molecules encoding MUC1 and MUC1/ECD wild-type ligands, such as neuregulin 2 isoforms 5 and 6. Inhibition of MUC1 expression will decrease the levels of MUC1/ECD available for binding to MUC1/ECD ligands. Inhibition of the expression of the endogenous ligands of MUC1/ECD will prevent or decrease the proliferative effect on cancer cells associated
20 with the binding of such ligands to MUC1/ECD. Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary sequences." By complementary, it is meant that polynucleotides are those capable of base-pairing according to the standard Watson-Crick complementary rules. The oligonucleotides of the present invention may be targeted wholly or in part to informational sequences, *i.e.*,
25 those coding for a protein, and other associated ribonucleotides such 5'-untranslated regions, 3'-untranslated regions, 5' cap regions and intron/exon junctions. Thus, the invention provides oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding MUC1 and/or MUC1/ECD wild-type ligands such as neuregulin 2 isoforms 5 and 6. The overall effect of interference with mRNA is
30 modulation of expression of neuregulin isoforms 5 and/or 6. Such modulation can be

measured in ways that are routine in the art. In addition, effects on cancer cell proliferation or tumor growth can be assessed.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment.

The antisense compounds in accordance with this invention preferably comprise from about 4 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 linked nucleobases. The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases. Examples of some preferred modified oligonucleotides include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages.

Another additional or alternative modification of the oligonucleotides of the present invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2'

position of the sugar of any nucleotide. The antisense compounds of the present invention also include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs.

The terms "specifically hybridizable" and "complementary" are used to indicate a degree of complementarity sufficient to result in stable and specific binding between the antisense oligonucleotide and the target nucleic acid sequence. An oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide considered "specifically hybridizable" when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility and decrease in expression of the product protein, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences.

The neuregulin 2 protein family comprises a number of alternatively spliced isoforms (Ring *et al.*, 1999). The coding sequences for neuregulin 2 isoforms 5 and 6 share the same nucleotide sequence from exons 1 through 6 of the neuregulin 2 gene that code for the first 416 amino acids of each protein but differ in the sequence coding for the carboxy terminal 10 amino acids of isoform 5 and for the carboxy terminal 6 amino acids of isoform 6. The coding DNA sequences of exons 1 through 6 are incorporated in SEQ ID NO: 10 through SEQ ID NO: 15 respectively. The sequences coding for the first 416 amino acids of isoforms 5 and 6 are nucleotides 313 through 1012 of SEQ ID NO: 10, nucleotides 51-222 of SEQ ID NO: 11, nucleotides 230-348 of SEQ ID NO: 12, nucleotides 100 through 220 of SEQ ID NO: 13, nucleotides 111 through 187 of SEQ ID NO: 14 and nucleotides 123 through 181 of SEQ ID NO: 15. The sequences coding for the carboxy terminals of isoform 5 and isoform 6 are nucleotides 132 through 164 of SEQ ID NO: 16 and nucleotides 30 through 50 of SEQ ID NO: 17 respectively.

As SEQ ID NO: 16 and SEQ ID NO: 17 are apparently not shared by other neuregulin gene products, in a preferred embodiment, the antisense oligonucleotide comprises a sequence of at least 4 nucleotides that is complementary to a region between nucleotides 132 and 164 of SEQ ID NO: 16 or nucleotides 30 through 50 of SEQ ID NO: 17. In a more preferred embodiment, the antisense oligonucleotides comprises a

sequence of at least 8 nucleotides that is complementary to a region between nucleotides 132 and 164 of SEQ ID. NO: 16 or nucleotides 30 through 50 of SEQ ID NO: 17.

In other embodiments, the antisense oligonucleotide comprises a sequence of at least 4 nucleotides that is complementary to a region between nucleotides 313 through 1012 of SEQ ID. NO 10, or a region between nucleotides 51-222 of SEQ ID. NO: 11, or a region between nucleotides 230-348 of SEQ ID NO: 12, or a region between nucleotides 100 through 220 of SEQ ID. NO: 13, or a region between nucleotides 111 through 187 of SEQ ID. NO: 14, or a region between nucleotides 123 through 181 of SEQ ID. NO: 15. In another embodiment the antisense oligonucleotide is at least 8 nucleotides that is complementary to a region of the one the foregoing nucleotides sequences.

In other embodiments, the antisense oligonucleotide comprises a sequence of at least 4 nucleotides, and preferably a sequence of at least 8 nucleotides, that is complementary to a non-coding region of SED ID NOS: 10 through 17.

In other embodiments of the invention, MUC1 directed antisense oligonucleotides comprise a sequence of at least 4 nucleotides that is complementary to SEQ ID NO: 18. In preferred embodiments, the antisense oligonucleotides comprises a sequence of at least 8 nucleotides that is complementary to SEQ ID NO: 18

The present invention also encompasses expression vectors comprising an expression control system that directs production of a transcript of the foregoing antisense oligonucleotides. In addition, the present invention provides for methods of hybridization comprising providing one of the foregoing antisense oligonucleotides and contacting such oligonucleotide with a nucleic acid comprising the target sequence under conditions that permit hybridization of the oligonucleotide with the nucleic acid. Also included are methods of inhibiting translation of mRNA comprising providing one of the foregoing antisense oligonucleotides and providing a cell comprising mRNA comprising the target sequence and introducing the oligonucleotide into the cell, wherein the oligonucleotide inhibits translation of the mRNA in the cell.

Another aspect of the present invention provides for pharmaceutical compositions comprising an antisense oligonucleotide of the invention and a pharmaceutically acceptable carrier.

VIII. Vaccines

The present invention also encompasses the use of MUC1/ECD peptides, *e.g.*, SEQ ID NO: 1 or fragments thereof, wherein such fragments comprise four or more consecutive amino acids of SEQ ID NO. 1, in a vaccine wherein the host mammal generates antibodies to the polypeptide which also act against the host's own MUC1/ECD. Vaccine preparation techniques are generally known in the art as described by Duffy (1980), and references cited therein, all of which are incorporated herein by reference.

The MUC1/ECD peptides may be conjugated to a carrier molecule such as a protein or Ficoll. The carrier protein is preferably one with a molecular weight of at least about 40,000 dalton and more preferably at least about 60,000 dalton. The vaccine formulation may comprise a pharmaceutically acceptable carrier and may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. Since the peptides or conjugates may be broken down in the stomach, the vaccine is preferably administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use.

IX. Formulations

The MUC1/ECD antagonists including binding inhibitors or oligonucleotides employed in the compositions and methods of the present invention can be formulated in a variety of conventional pharmaceutical formulations and administered to cancer patients, in need of treatment, by any one of the drug administration routes conventionally employed including oral, intravenous, intraarterial, parental or intraperitoneal.

For oral administration the compositions of the present invention may be formulated, for example, with an inert diluent or with an assimilable edible carrier, or enclosed in hard or soft shell gelatin capsules, or compressed into tablets, or incorporated

directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations may, of course, be varied and may conveniently be
5 between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, a gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium
10 phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit for is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be
15 present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing a dosage unit form should be pharmaceutically
20 pure and substantially non-toxic in the amounts employed. In addition, other chemotherapeutic compounds may be incorporated into sustained-release preparation and formulations.

In regard to formulations comprising oligonucleotides, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the
25 oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure.

Pharmaceutical formulations of the compositions of the present invention which are suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that each syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the compositions of the present invention in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the

therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the composition.

X. Treatment Methods

5 Tumors that can be suitably treated with the methods of the present invention include; but are not limited to, tumors of the brain (glioblastomas, medulloblastoma, astrocytoma, oligodendroglioma, ependymomas), lung, liver, spleen, kidney, lymph node, small intestine, pancreas, blood cells, colon, stomach, breast, endometrium, prostate, testicle, ovary, skin, head and neck, esophagus, bone marrow, blood and other tissue. The
10 tumor may be distinguished as metastatic and non-metastatic. Pre-malignant lesions may also be suitably treated with the methods of the present invention.

The treatment with the MUC1/ECD antagonists of the present invention may precede or follow irradiation and/or chemotherapy by intervals ranging from seconds to weeks and/or be administered concurrently with such treatments. In embodiments where
15 the MUC1/ECD antagonists and irradiation and/or chemotherapy are applied separately to the cell, steps should be taken to ensure that a significant period of time does not expire between the time of each delivery, such that the combination of the two or three treatments would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with the treatment
20 agents or modalities within amount 0.1 to 25 h of each other and, more preferably, within about 1 to 4 h of each other, with a delay time of only about 1 h to about 2 h being most preferred. In some situations, it is desirable to extend the time period of treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) or several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations. In any case, the invention
25 contemplates that the MUC1/ECD antagonists may be given before, after or even simultaneously with the ionizing radiation and/or chemotherapeutic agent.

Some chemotherapeutic agents transiently induce MUC1 expression on cancer cells 0.5 to 12 hours after contact of the carcinoma cells with the chemotherapeutic agents. Thus, in some embodiments the administration of MUC1/ECD binding
30 inhibitors, especially antibodies to the MUC/ECD sequence SEQ ID NO. 1, optionally

conjugated to a toxin or radionucleotide, is coordinated with the increased expression of MUC1 on the cancer cells. In other embodiments, agents other than chemotherapeutic agents may be used to increase MUC1 expression prior to treatment with a MUC1 binding inhibitor, especially antibodies to the MUC/ECD sequence SEQ ID NO. 1, 5 optionally conjugated to a toxin or radionucleotide.

In the methods of the present invention, the actual dosage of MUC1/ECD antagonists employed will depend on a variety of factors including the type and severity of cancer being treated, and the additive or synergistic treatment effects of the MUC1/ECD antagonists and the other treatment modality or modalities selected. 10

EXAMPLES OF THE INVENTION

Example 1: Peptides

The MUC1/ECD polypeptide sequence, as typically found in MUC1-expressing cells, is provided by SEQ ID NO: 1. A number of polypeptide sequences have been synthesized by standard techniques. These include peptides P1 (SEQ ID NO: 2), P2 15 (SEQ ID NO: 5) and P3 (SEQ ID NO: 6), which are polypeptide fragments of MUC1/ECD. P1 (SEQ ID NO: 2) represents amino acids 5 through 20 of MUC1/ECD (SEQ ID NO: 1) with a cysteine added at the carboxy terminal. P2 (SEQ ID NO: 5) represents amino acids 13 through 28 of MUC1/ECD (SEQ ID NO: 1) with a cysteine added at the carboxy terminal. P3 (SEQ ID NO: 6) represents amino acids 27 through 44 20 of MUC1/ECD (SEQ ID NO: 1) with a cysteine at the carboxy terminal. In addition, the synthesized polypeptide sequence SEQ ID NO: 7 incorporates amino acids 6 through 24 of MUC1/ECD (SEQ ID NO: 1) with a cysteine added at the carboxy terminal. The synthesized polypeptide P0 (SEQ ID NO: 8) incorporates a 19 amino acid sequence 25 occurring in the MUC1 protein occurring just prior to the amino terminus of MUC1/ECD and represents the a potential cleavage site. A cysteine was again added at the carboxy terminal of the sequence at it occurs in the MUC 1 sequence.

Example 2: Anti-MUC1-P1 Antibody**A. Generation of Antibody**

The polypeptide P1 (SEQ. ID NO: 4), contains the QYK motif and other sequences homologous to ligand binding domains of cytokine receptors (Zrihan-Licht, *et al.*, 1994). A polyclonal antibody was raised in rabbits against polypeptide P1 (SEQ ID NO. 4) conjugated to KLH. Serum was prepared by standard methods.

Polyclonal antibodies were also raised against the polypeptide SEQ ID NO: 7, whereby the immunogen was formed by conjugating the polypeptide SEQ ID NO: 7 to KLH. Antibodies have been obtained from 2 rabbits, designated 3402-1 and 3402-2. Both serum and affinity purified antibody preparations have been prepared by standard methodologies.

B. Stimulation of Human Carcinoma Cells by Anti-MUC1-P1-Antibody

Human ZR-75-1 carcinoma cells were grown to 80% confluence in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and then passed onto a 6-well plate at 1×10^4 cells per well. After overnight starvation in medium containing 0.1% FBS, anti-MUC1-P1 antibody was added to each well at the amounts indicated in FIG. 1 and incubated for 48 hours. Cell numbers were quantified after another 3 days of incubation in the presence of 0.1 % FBS. As shown in FIG. 1, anti-MUC1-P1 stimulates the growth of ZR-75-1 cells in a dose dependent fashion.

To assess the specificity of anti-MUC1-P1 antibody stimulation, human MUC1-negative SW480 colon cancer cells were stably transfected to express empty vector (SW480/V) or MUC1 (SW480/MUC1). SW80 colon cancer cells were transfected with either pCMV-IE-ak1-dhfr vector or pCMV-IE-ak1-dhfr-MUC1 using lipofectamide (Ligtenburger *et al.*, 1992). Cells were grown in the presence of 800 $\mu\text{g/ml}$ G418 (neomycin) and serially diluted to single-cell populations. Single cell clones that express MUC1 (SW480/MUC1) were selected. Both SW480 cells types were grown to 80 % confluence in DMEM containing 10% FBS and plated onto 6-well plates at 5×10^4 cells per well. After overnight starvation in medium containing 0.1% FBS, anti-MUC1-P1 antibody was added at the indicated concentrations and incubated for 48 hr. Cell numbers were quantified after a further day of incubation (3 days total). As shown in FIG. 2, anti-

MUC1-P1 stimulates the growth of SW480/MUC1 cells but not SW48-0/V cells. These findings confirm that the anti-MUC1-P1 antibody stimulates the growth of human carcinoma cells by specifically interacting with MUC1.

5 Example 3: Endogenous MUC1 ECD Ligands

The finding that anti-MUC1-P1 stimulates growth of human carcinoma cells is suggestive of the potential existence of natural MUC1 ECD ligands. To investigate this potential, ZR-75-1 cells were screened as a possible source of MUC1 ligand(s). Conditioned medium was prepared by culturing ZR-75-1 cells in RPMI 1640 medium containing 0.1% FBS for 72 hr and then preparing a supernatant. The conditioned medium was added to SW480/V and SW480/MUC1 cells that were growth arrested in DME containing 0.1% FBS as previously described. Conditioned medium was added at the concentration indicated in Fig. 3 and the cells were maintained for 3 days prior to quantification of cell numbers. As shown in FIG. 3, ZR-75-1 cells express a soluble ligand that stimulates carcinoma cell growth by binding to MUC1.

To identify the soluble MUC1 ligand(s), a fusion protein comprising the MUC1/ECD (SEQ ID. No: 1) and glutathione S-transferase (GST) was prepared. GST was amplified by PCR using a set of primers as follows:

5'-ATTAGGCTAGCCTGGTTCCGCGTGGTTCTATGTCCCCTATACTAGGTTA-3',
and 5'-CAAGGGGATCCCTACGGAACCAGATCCGATTTTGG-3', and inserted between the NheI and BamHI sites of pET-11d (the "pET-11d-GST vector"). MUC1/ECD was amplified by PCR using a set of primers as follows:
5'-TCTGGCCATGGGAGAAGGTACCATCAAT-3', and
5'-AGCGCGCTAGCCCAGCCTGGCACCCCAGC-3', and inserted between the NcoI and NheI sites of pET11d-GST vector (the "pET11d-GST-MUC1/ECD" vector).

The GST fusion protein was prepared by incubating logarithmically growing E. coli BL21(D3)pLysS cells transformed with pET11d-GST-MUC1/ECD or pET11d-GST with 0.1 mM isopropyl- β -D-thiogalactopyranoside for 6 hr at 25 °C. Cells were pelleted and resuspended in PBS containing 20 % sucrose, 5 mM MgCl₂, 0.5 % NP-40, then sonicated. Debris was removed by centrifugation at 10,000 x g for 30 min. at 4 °C. The

supernatant was applied to bulk glutathione sepharose 4B and incubated for 4 hr at 4 °C prior to washing and packing into a column. The fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl, pH 9.5. The purified fusion protein was dialyzed with PBS.

5 Cytosolic fractions of cultured cells were prepared by harvesting ZR75-1 cells in PBS containing 40mM EDTA. After washing with PBS, cells were resuspended with ice-cold homogenized buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA and protease inhibitors cocktail (Roche) and put on ice for 15 min. The suspension was homogenized with a Dounce
10 homogenizer. The homogenate was centrifuged at 1,00 x g for 5 min at 4 °C. The supernatant was collected as a cytosol fraction and stored at 0 °C.

The GST-MUC1/ECD fusion protein (1.8 mg) was immobilized on 400 µl of glutathione-sepharose 4B, which was packed into a column and equilibrated with buffer A (30 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol). The
15 cytosolic fraction was first precleared by passing it through a glutathione-sepharose 4B column and was then loaded onto the GST-MUC1/ECD affinity column which was then washed twice with 2 x 10 ml of buffer A. The protein bound to the column was eluted by the addition of 2 ml of buffer B (buffer A containing 0.15 M NaCl), and fractions of 0.4 ml each were collected. The second and third fractions were mixed and loaded on
20 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As a control, the GST protein (1.5 mg) was immobilized on 400 µl of glutathione-sepharose 4B and the experiment performed as described above. The results demonstrated that MUC1/ECD binds to a 45 kDa protein. A similar experiment was performed with lysates of human MCF-7 breast carcinoma cells confirmed the binding of MUC1/ECD with a 45 kDa
25 protein.

The 45 kDa from the GST-MUC1/ECD adsorbate was excised from the gel, dehydrated with acetonitrile, and then rehydrated with 100 mM ammonium bicarbonate. The gel pieces were then suspended in 12.5 ng/µl trypsin/50 mM ammonium bicarbonate. Digestion was carried out at 37 °C for 10-12 hr. The masses of the trypsin-digested

peptides were analyzed by matrix assisted laser desorption/ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) using a Voyager DE-PRO (Perceptive Biosystem Inc., Framingham, MA). Two related proteins, designated ML-1 and ML-2, were identified by mass fingerprinting. The sequences of ML-1 (SEQ ID NO: 3) and ML-2 (SEQ ID NO: 4) are as those previously disclosed for two neuregulin isoforms, NRG2 splice isoform 5 and NRG2 splice isoform 6 respectively.

Example 4: MUC1 as an Oncogene

A. MUC1 supports growth in soft agar

The expression of MUC1 was shown to be functionally significant regarding the exhibition of the malignant phenotype. Cell lines that stably express MUC1 were generated. HCT116 colon cancer cells were transfected with pIRES-puro2 vector or pIRES-puro2-MUC1 by lipofectamine and selected for puromycin-resistance. Studies were performed with human MUC1-negative HCT116 colon cancer cells with single clones that stably express either the empty vector (VCT116/V) or MUC1 (HCT116/MUC1). The HCT 116/V and HCT116/MUC1 cells were assayed for anchorage-independent growth in soft agar. Cells (1×10^5 / 60 mm dish) were suspended in 0.33% agarose-containing DMEM medium supplemented with 10 % FBS and layered over an agarose plug (0.5 % agarose in DMEM supplemented with 10 % FBS). The cells were incubated to 4 weeks, during which time fresh medium was added to the plates every week. Colonies larger than 70 μ m in diameter were counted after 4 weeks. Expression of wild-type MUC1 was associated with a marked increase in the size and number of colonies compared to that obtained with HCT/116/V cells. These findings were confirmed by similar studies performed with SW480/V and SW480/MUC1 cells wherein MUC1 expression was again shown to support anchorage-independent growth of SW480 cells.

B. MUC1 supports human tumor formation in nude mice

To assess the effects of MUC1 on human tumor growth in vivo. Five to six week old athymic, Balbc/nu/nu mice (Taconic, Germantown, NY) were injected subcutaneously in the right flank with 1×10^6 HCT116/V or HCT 116/MUC1 cells.

Tumors (4 mice/group) were measured twice a week. Tumor volumes were calculated by the following formula: $\frac{1}{2}(\text{length} \times \text{width}^2)$. Experiments were terminated when tumor volume exceeded 2 cm³. Measurements of tumor volume over time demonstrated little HCT116/V cell growth. By comparison there was a marked increase in the growth of HCT116/MUC1 tumors.

Example 5: MUC1 Expression is Induced by Oxidative and Genotoxic Stress

To determine whether MUC1 is induced in response to oxidative stress, MCF-7 cells were treated with hydrogen peroxide. Cell lysates were analyzed by immunoblotting with anti-DF3/MUC1 antibody. Lysates were prepared by suspending MCF-7 cells in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM PMSF, 3 mM NaF, 1 mM sodium vanadate, 1mM DTT with protease inhibitors and either 1% NP-40 or 1% Brij-96) for 30 min. on ice. Lysates were cleared by centrifugation and equal amounts of proteins were resolved by SDS-PAGE. Proteins were then transferred to nitrocellulose filters, blocked by incubation in 5 % non-fat dry milk in PBS with 0.05 % Tween-20 and probed with anti-MUC1 antibody (Pandey *et al.*, 1995). Anti-actin was used as a control. The results demonstrated that MUC1 is rapidly and transiently induced in response to oxidative stress while there was no effect of hydrogen peroxide on levels of actin expression.

Similar studies were performed with genotoxic agents. The results demonstrate that treatment of MCF-7 cells with daunorubicin is associated with a rapid and transient induction of MUC1, and not actin, expression. The available evidence indicates that MUC1 is induced by diverse cytotoxic agents, including taxol, cisplatin and ionizing radiation.

Example 6: MUC1 Inhibits the Apoptotic Response to Oxidative and Genotoxic Stress

To determine whether MUC1 inhibits the apoptotic response to oxidative stress, HeLa cells stably expressing the empty vector or MUC1 were treated with 1 mM hydrogen peroxide for 1 hr. DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Beckton Dickerson). Numbers of

cells with sub-G1 DNA content were determined with a MODFIT LT program, (Verity Software House, Topsham, ME) (Yuan *et al.*, 1997). The cells were analyzed for induction of sub-G1 DNA by flow cytometry as a marker of apoptosis. As shown in FIG. 4, the results demonstrate that MUC1 inhibits the apoptotic response to oxidative stress.

5 The induction of apoptosis in cells treated with 0.01 mM taxol for 20 hr was assessed by measuring sub-G1 DNA content of HeLa cells expressing empty vector or MUC1. As shown in FIG. 4, as with oxidative stress, taxol-induced apoptosis was inhibited by MUC1 expression.

10 **Example 7: Effect of NM-3 on MUC1 Expression**

MCF-7 cells were treated with NM-3 (2-(8-hydroxy-6-methoxy-1-oxo-1 *H*-2-benzopyran-3-yl) propionic acid) at 100-400 µg/ml for 48 hr. DF3 antigen levels were visualized by immunoblot analysis with DF3 MAb (Kufe, U.S. Patent 5,506,343, herein incorporated by reference).

15 A decrease in the intracellular levels of DF3 antigen of NM-3 treated cells relative to non-treated cells was observed. Similar results were found in ZR-75-1 and BT-20 cell lines. The NM-3 mediated decrease of intracellular DF3 antigen was shown to be both dose- and time dependent. To determine whether NM-3 impaired the extracellular localization of the MUC1 DF3 antigen, the antigen levels in cell culture medium
20 supernatants and on MCF-7 cells after NM-3 treatment were investigated. The levels of DF3 antigen were reduced in both localizations. These findings suggest that NM-3 inhibits MUC1 protein expression.

Hybridization studies were performed to determine whether the effect of NM-3 on MUC1-expression was detectable at the transcriptional level. A ³²P-laballed DF3 DNA
25 probe hybridized to two transcripts of 4.5 and 7.0 kb in total cellular RNA after NM-3 treatment on MCF-7 cells for 48 hr. The levels of both mRNA were decreased relative to controls wherein MCF-7 cells were incubated in the absence of NM-3. The same results were observed using ZR-75-1 and BT-20 cell lines. These findings suggest that DF3 expression is regulated at the transcriptional level after NM-3 treatment on those cell
30 lines.

To determine whether NM-3 inhibits the expression of cellular surface proteins as well, the level of epidermal growth factor receptor (EGF-R) expression after NM-3 treatment was tested on MCF-7, ZR-75-1 and BT-20 cell lines. Compared to controls wherein cells were incubated in the absence of NM-3, there were no detectable changes in
5 EGF-R expression after NM-3 treatment. These results indicate a selective effect of NM-3 on MUC1 expression without inhibition of surface molecular expression.

The present invention has been shown by both description and examples. The Examples are only examples and cannot be construed to limit the scope of the invention. One of ordinary skill in the art will envision equivalents to the inventive process
10 described by the following claims that are within the scope and spirit of the claimed invention.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

- 5 Barry & Sharkey, Hum. Pathol., 16:225-7, 1985.
Brunner *et al.*, Nature, 385:829-833, 1997.
Daniels & Reynolds, Mol. Cell. Biol., 19:3614-23, 1999.
Dawson *et al.*, Science 266:776, 1994.
Duffy, in Vaccine Preparation Techniques, Noyes Data Corporation of Park Ridge, N.J.,
10 1980.
Finn *et al.*, Immunol. Rev. 145:61-89, 1995.
Gansow, Int. J. Rad Appl. Instrum. [B], Nucl. Med. Biol. 18:369-381, 1991.
Goding, In: Monoclonal Antibodies: Principles and Practice, 2d ed., Orlando, Fla., Academic Press, pp. 60-61, 1986.
- 15 Itzkowitz *et al.*, Cancer, 66:1960-6, 1990.
Jawhari *et al.*, J. Pathol., 189:180-85, 1999.
Jones *et al.*, Nature 321:522-525, 1986.
Kufe *et al.*, Hybridoma, 3:223-232, 1984.
Li *et al.*, Mol Cell Biol., 18:7216-24, 1998.
- 20 Li *et al.*, J. Biol. Chem., 276:6061-64, 2001.
Li *et al.*, J. Biol Chem, e-publication manuscript C100359200, August 1, 2001(a).
Li & Kufe, Biochem Biophys. Res. Commun., 281, 440-43, 2001.
Ligtenburger *et al.*, Cancer Res., 15:2318-2324, 1992.
Molenaar *et al.*, Cell, 86:391-399, 1996.
- 25 Novak & Dedhar, Cell Mol. Life Sci., 523-37, 1999.
Olsnes & Pihl, Pharmac. Ther. 25:355-381, 1981.
Pandey *et al.*, Cancer Res., 55:4000-4003, 1995.
Pastan *et al.*, Ann. Rev. Biochem. 61:331-354, 1992.
Perey *et al.*, Cancer Res., 52:2563-68, 1992.

- Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113:269-315, Rosenberg and Moore eds. Springer-Verlag, New York, 1994.
- Presta, *Curr. Op. Struct Biol.* 2:593-596, 1992.
- Reddish *et al.*, *Cancer Immunol. Immunother.*, 42:303-9, 1996.
- 5 Reynolds *et al.*, *Mol. Cell. Biol.* 9:629-38, 1989.
- Reynolds *et al.*, *Mol. Cell. Biol.*, 14:8333-41, 1994.
- Riechmann *et al.*, *Nature* 332:323-329, 1988.
- Ring *et al.*, *Human Genet.*, 104:326-32, 1999.
- Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor
- 10 Laboratory Press, Cold Spring Harbor, N.Y. 1989.
- Shimazui *et al.*, *Cancer Res.*, 56:4154-58, 1996.
- Smith & Waterman, *Adv. Appl. Math.*, 2:482-489, 1981.
- Strouss & Decker, *Crit. Rev. Biochem. Mol. Biol.*, 27:57-92, 1992.
- Takeichi, *Annu. Rev. Biochem.*, 59:237-52, 1990.
- 15 Thorpe *et al.*, *Immunological Rev.* 62:119-158, 1982.
- Thorpe *et al.*, *Monoclonal Antibodies in Clinical Medicine*, pp. 168-190 Academic Press, NY 1982(a).
- Waldmann, *Science*, 252:1657, 1991.
- Yamamoto *et al.*, *J. Biol. Chem.* 272:12492-94, 1997.
- 20 Yuan *et al.*, *PNAS*, 94:1437-1440, 1997.
- Zrihan-Licht, *et al.*, *FEBS Let.*, 356:130-36, 1994.
- U.S. Pat. No. 4,675,382
- U.S. Pat. No. 4,894,443,
- U.S. Pat. No. 5,506,343

WHAT IS CLAIMED IS:

1. The use of a MUC1 extracellular domain antagonist for the manufacture of a medicament to inhibit the proliferation of MUC1-expressing cancer cells.
2. The use of a MUC1 extracellular domain antagonist of claim 1, wherein said MUC1 extracellular domain antagonist downregulates the expression of the MUC1 extracellular domain.
3. The use of a MUC1 extracellular domain antagonist of claim 1, wherein said MUC1 extracellular domain antagonist is a MUC1 extracellular domain binding inhibitor.
4. The use of a MUC1 extracellular domain antagonist of claim 3, wherein said MUC1 extracellular domain binding inhibitor is the polypeptide of SEQ ID NO: 1, or a fragment comprising at least four consecutive amino acids of SEQ ID NO: 1, or conservative variants thereof.
5. The use of a MUC1 extracellular domain antagonist of claim 3, wherein said MUC1 extracellular domain binding inhibitor is the polypeptide of SEQ ID NO: 4 or SEQ ID NO: 5 or conservative variants thereof.
6. The use of a MUC1 extracellular domain antagonist of claim 3, wherein said MUC1 extracellular domain binding inhibitor is an antibody or fragment thereof that binds to an epitope within sequences of the peptides selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.
7. The use of a MUC1 extracellular domain antagonist of claim 3, wherein said MUC1 extracellular domain binding inhibitor is an antibody or fragment thereof that binds to an epitope within sequence of SEQ ID NO: 1.

8. The use of a MUC1 extracellular domain antagonist of claim 6, wherein said antibody is a monoclonal antibody.
9. The use of a MUC1 extracellular domain antagonist of claim 8, wherein said monoclonal antibody is humanized.
10. The use of a MUC1 extracellular domain antagonist of claim 8, wherein said monoclonal antibody is a human antibody.
11. The use of a MUC1 extracellular domain antagonist of claim 6, wherein said antibody is a bispecific antibody.
12. The use of a MUC1 extracellular domain antagonist of claim 6, wherein said fragment comprises an antigen binding region.
13. The use of a MUC1 extracellular domain antagonist of claim 6, wherein said antibody or fragment thereof is conjugated to a chemotherapeutic agent, radioisotope, toxin, or an effector that induces a cytolytic or cytotoxic immune response.
14. The use of a MUC1 extracellular domain antagonist of claim 13, wherein said antibody or fragment thereof is conjugated to a cytokine, an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic, an alkylating agent, a naturally derived toxin, or an Fc region of a IgG1 immunoglobulin.
15. The use of a MUC1 extracellular domain antagonist of claim 1, wherein said effective amount of a MUC1 extracellular antagonist is administered as a composition comprising said MUC1 extracellular domain antagonist and a pharmaceutically acceptable carrier.

16. The use of a MUC1 extracellular domain antagonist of claim 1, further comprising administration of a chemotherapeutic agent or radiation.
17. A use of a MUC1 extracellular domain antagonist for the manufacture of a medicament for use in combination with a chemotherapeutic agent or radiation for the treatment of cancer.
18. The use of a MUC1 extracellular domain antagonist of claim 17, wherein said MUC1 extracellular domain antagonist downregulates the expression of the MUC1 extracellular domain.
19. The use of a MUC1 extracellular domain antagonist of claim 17, wherein said MUC1 extracellular domain antagonist is a MUC1 extracellular domain binding inhibitor.
20. The use of a MUC1 extracellular domain antagonist of claim 19, wherein said MUC1 extracellular domain binding inhibitor is the polypeptide of SEQ ID NO: 1, or a fragment comprising at least four consecutive amino acids of SEQ ID NO: 1, or conservative variants thereof.
21. The use of a MUC1 extracellular domain antagonist of claim 19, wherein said MUC1 extracellular domain binding antagonist is the polypeptide of SEQ ID NO: 4 or SEQ ID NO: 5 or conservative variants thereof.
22. The use of a MUC1 extracellular domain antagonist of claim 19, wherein said MUC1 extracellular domain binding inhibitor is an antibody or fragment thereof that binds to an epitope within sequences of the peptides selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

23. The use of a MUC1 extracellular domain binding antagonist of claim 19, wherein said MUC1 extracellular domain binding antagonist is an antibody or fragment thereof that binds to an epitope within sequence of SEQ ID NO: 1.
24. The use of a MUC1 extracellular domain antagonist of claim 22, wherein said antibody is a monoclonal antibody.
25. The use of a MUC1 extracellular domain antagonist of claim 24, wherein said monoclonal antibody is humanized.
26. The use of a MUC1 extracellular domain antagonist of claim 24, wherein said monoclonal antibody is a human antibody.
27. The use of a MUC1 extracellular domain antagonist of claim 22, wherein said antibody is a bispecific antibody.
28. The use of a MUC1 extracellular domain antagonist of claim 22, wherein said fragment comprises an antigen binding region.
29. The use of a MUC1 extracellular domain antagonist of claim 22, wherein said antibody or fragment thereof is conjugated to a chemotherapeutic agent, radioisotope, toxin, or an effector that induces a cytolytic or cytotoxic immune response.
30. The use of a MUC1 extracellular domain antagonist of claim 29, wherein said antibody or fragment thereof is conjugated to a cytokine, an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic, an alkylating agent, a naturally derived toxin, or an Fc region of a IgG1 immunoglobulin.
31. The use of a MUC1 extracellular domain antagonist of claim 17, wherein said chemotherapeutic agent is selected from the group consisting of alkylating agents,

topoisomerase inhibitors, antimetabolites, tubulin interactive agents, anti-hormonal agents, ornithine decarboxylase inhibitors and tyrosine kinase inhibitors.

32. A pharmaceutical composition comprising the polypeptide of SEQ ID NO: 1, or a fragment comprising at least four consecutive amino acids of SEQ ID. NO: 1, or conservative variants thereof, and a pharmaceutically acceptable carrier.

33. The composition of claim 32, wherein said polypeptide is the polypeptide of SEQ ID NO: 5, or conservative variants thereof.

34. A pharmaceutical composition comprising the polypeptide of SEQ ID NO: 4 or conservative variants thereof, and a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising an antibody or fragment thereof that binds to an epitope within sequences of the peptides selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 and a pharmaceutically acceptable carrier.

36. The composition of claim 35, wherein said antibody or fragment thereof binds to an epitope within sequence of SEQ ID NO: 1.

37. The composition of claim 36, wherein said antibody is a monoclonal antibody.

38. The composition of claim 37, wherein said monoclonal antibody is humanized.

39. The composition of claim 37, wherein said monoclonal antibody is a human antibody.

40. The composition of claim 35, wherein said antibody is a bispecific antibody.

41. The composition of claim 35, wherein said fragment comprises an antigen binding region.

42. The composition of claim 35, wherein said antibody or fragment thereof is conjugated to a chemotherapeutic agent, radioisotope, toxin, or an effector that induces a cytolytic or cytotoxic immune response.

43. The composition of claim 42, wherein said antibody or fragment thereof is conjugated to a cytokine, an antimetabolite, an anthracycline, a vinca alkaloid, an antibiotic, an alkylating agent, a naturally derived toxin, or an Fc region of a IgG1 immunoglobulin.

44. A method of identifying a compound the inhibits the binding of ligands to the extracellular domain of MUC1, the method comprising:

- (a) providing a polypeptide comprising the polypeptide of SEQ ID. NO: 1 or the polypeptide of SEQ ID NO: 5;
- (b) contacting said polypeptide test compound and an antibody that binds to an epitope within SEQ ID NO: 4;
- (c) determining whether the binding of said antibody that binds to an antibody to an epitope within SEQ ID NO: 4 to the polypeptide SEQ ID. NO: 1 or the polypeptide of SEQ ID NO: 5 is decreased relative to an appropriate control.

45. A pharmaceutical composition comprising a compound identified by the method of claim 44 and a pharmaceutically acceptable carrier.

46. A method of identifying a compound that inhibits the proliferation of MUC1-expressing cancer cells, the method comprising:

- (a) providing a population of MUC1-expressing cancer cells;
- (b) contacting said population of MUC1-positive cancer cells with a test compound and an antibody that binds to an epitope within SEQ ID NO: 5;

(c) determining whether the proliferation of said population of MUC1-expressing cancer cells is decreased by comparison to an appropriate control.

47. A pharmaceutical composition comprising a compound identified by the method of claim 46 and a pharmaceutically acceptable carrier.

48. A method of identifying a compound that downregulates the expression the extracellular domain of MUC1, the method comprising:

- (a) providing a population of MUC1-expressing cancer cells;
- (b) contacting said population of MUC1-expressing cancer cells with a test compound;
- (c) utilizing an anti-MUC1/ECD antibody to identify polypeptides comprising MUC1/ECD in the MUC1/ECD-expressing cancer cells; and
- (d) determining whether the expression of polypeptides comprising MUC1/ECD is decreased in comparison to controls wherein the test compound was excluded.

49. The use of a NM-3 for the manufacture of a medicament to downregulate the expression of the extracellular domain of MUC1/ECD in a cancer.

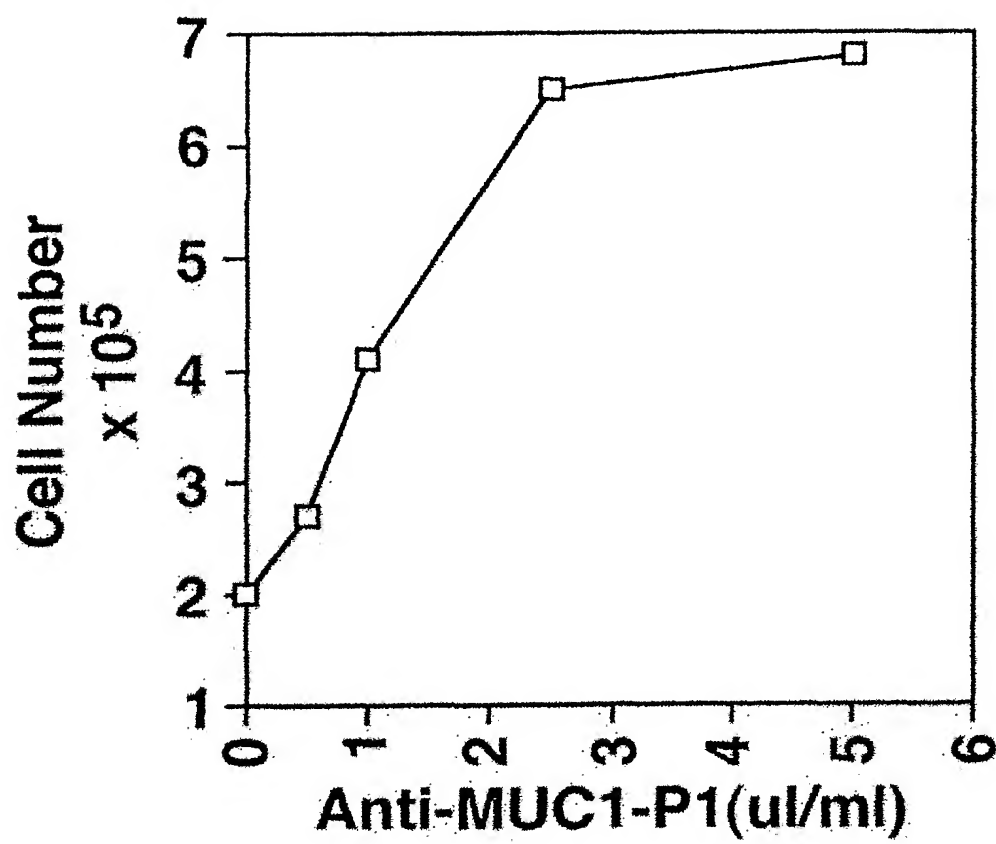


FIG. 1

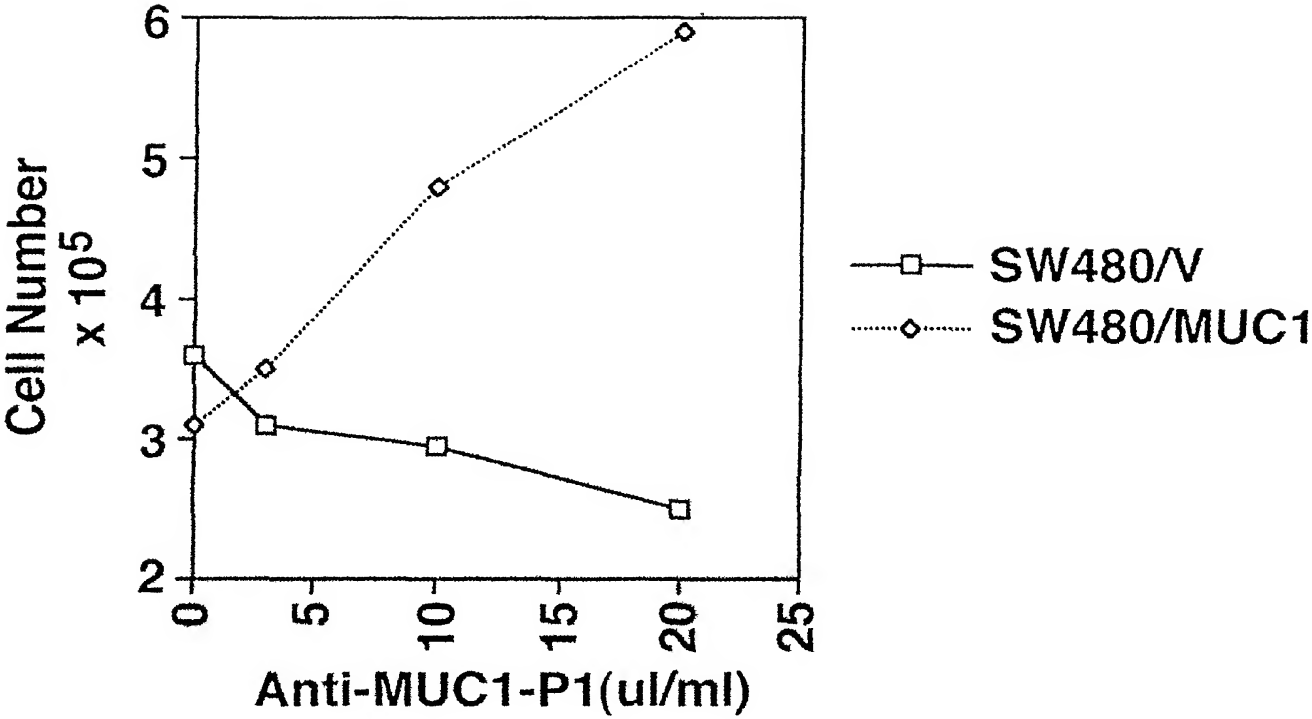


FIG. 2

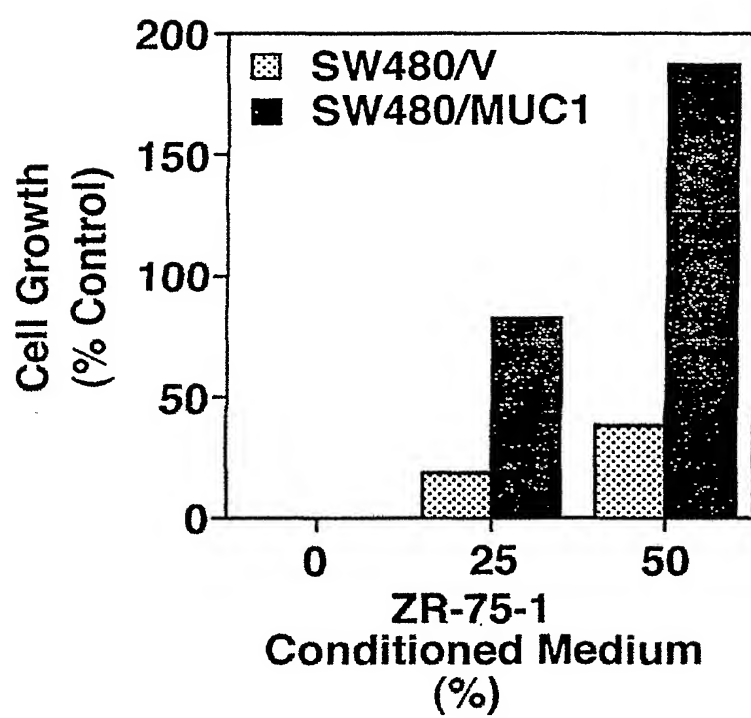


FIG. 3

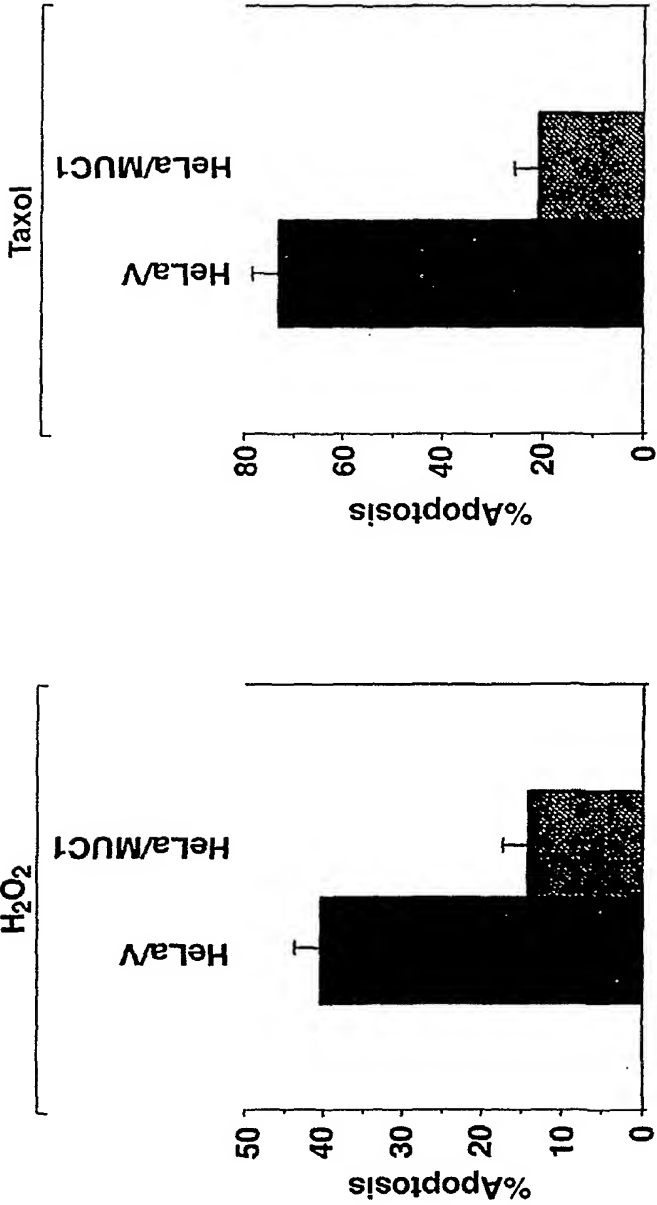


FIG. 4

SEQUENCE LISTING

<110> Kufe, Donald W.
Ohno, Tsuneya

<120> MUC1 EXTRACELLULAR DOMAIN AND CANCER TREATMENT COMPOSITIONS AND METHODS DERIVED THEREFROM

<130> MUC1 APPLICATION

<150> 60/231,841

<151> 2000-09-11

<160> 18

<170> PatentIn version 3.1

 $\langle 210 \rangle$ 1

<211> 45

<212> PRT

<213> Homo sapiens

<400> 1

Thr Ile Asn Val His Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr
1 5 10 15

Glu Ala Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp Val Ser Val Ser
20 25 30

Asp Val Pro Phe Pro Phe Ser Ala Gln Ser Gly Ala Gly
35 40 45

 $\langle 210 \rangle$ 2

<211> 426

<212> PRT

<213> Homo sapiens

<400> 2

Met Arg Gln Val Cys Cys Ser Ala Leu Pro Pro Pro Pro Leu Glu Lys
 1 5 10 15

Gly Arg Cys Ser Ser Tyr Ser Asp Ser Ser Ser Ser Ser Ser Glu Arg
 20 25 30

Ser Ser Ser Ser Ser Ser Ser Ser Ser Glu Ser Gly Ser Ser Ser Arg
 35 40 45

Ser Ser Ser Asn Asn Ser Ser Ile Ser Arg Pro Ala Ala Pro Pro Glu
 50 55 60

Pro Arg Pro Gln Gln Gln Pro Gln Pro Arg Ser Pro Ala Ala Arg Arg
 65 70 75 80

Ala Ala Ala Arg Ser Arg Ala Ala Ala Ala Gly Gly Met Arg Arg Asp
 85 90 95

Pro Ala Pro Gly Phe Ser Met Leu Leu Phe Gly Val Ser Leu Ala Cys
 100 105 110

Tyr Ser Pro Ser Leu Lys Ser Val Gln Asp Gln Ala Tyr Lys Ala Pro
 115 120 125

Val Val Val Glu Gly Lys Val Gln Gly Leu Val Pro Ala Gly Gly Ser

130	135	140															
Ser Ser Asn Ser Thr Arg Glu Pro Pro Ala Ser Gly Arg Val Ala Leu																	
145	150	155	160														
Val Lys Val Leu Asp Lys Trp Pro Leu Arg Ser Gly Gly Leu Gln Arg																	
	165	170	175														
Glu Gln Val Ile Ser Val Gly Ser Cys Val Pro Leu Glu Arg Asn Gln																	
	180	185	190														
Arg Tyr Ile Phe Phe Leu Glu Pro Thr Glu Gln Pro Leu Val Phe Lys																	
	195	200	205														
Thr Ala Phe Ala Pro Leu Asp Thr Asn Gly Lys Asn Leu Lys Lys Glu																	
	210	215	220														
Val Gly Lys Ile Leu Cys Thr Asp Cys Ala Thr Arg Pro Lys Leu Lys																	
	225	230	235	240													
Lys Met Lys Ser Gln Thr Gly Gln Val Gly Glu Lys Gln Ser Leu Lys																	
	245	250	255														
Cys Glu Ala Ala Ala Gly Asn Pro Gln Pro Ser Tyr Arg Trp Phe Lys																	
	260	265	270														
Asp Gly Lys Glu Leu Asn Arg Ser Arg Asp Ile Arg Ile Lys Tyr Gly																	
	275	280	285														

Asn Gly Arg Lys Asn Ser Arg Leu Gln Phe Asn Lys Val Lys Val Glu
 290 295 300

Asp Ala Gly Glu Tyr Val Cys Glu Ala Glu Asn Ile Leu Gly Lys Asp
 305 310 315 320

Thr Val Arg Gly Arg Leu Tyr Val Asn Ser Val Ser Thr Thr Leu Ser
 325 330 335

Ser Trp Ser Gly His Ala Arg Lys Cys Asn Glu Thr Ala Lys Ser Tyr
 340 345 350

Cys Val Asn Gly Gly Val Cys Tyr Tyr Ile Glu Gly Ile Asn Gln Leu
 355 360 365

Ser Cys Lys Cys Pro Asn Gly Phe Phe Gly Gln Arg Cys Leu Glu Lys
 370 375 380

Leu Pro Leu Arg Leu Tyr Met Pro Asp Pro Lys Gln Ser Val Leu Trp
 385 390 395 400

Asp Thr Pro Gly Thr Gly Val Ser Ser Ser Gln Trp Ser Thr Ser Pro
 405 410 415

Lys Pro Arg Ser Cys Thr Arg Arg Gly Ser
 420 425

<210> 3

<211> 422

<212> PRT

<213> Homo sapiens

<400> 3

Met Arg Gln Val Cys Cys Ser Ala Leu Pro Pro Pro Pro Leu Glu Lys
 1 5 10 15

Gly Arg Cys Ser Ser Tyr Ser Asp Ser Ser Ser Ser Ser Ser Glu Arg
 20 25 30

Ser Ser Ser Ser Ser Ser Ser Ser Ser Glu Ser Gly Ser Ser Ser Arg
 35 40 45

Ser Ser Ser Asn Asn Ser Ser Ile Ser Arg Pro Ala Ala Pro Pro Glu
 50 55 60

Pro Arg Pro Gln Gln Gln Pro Gln Pro Arg Ser Pro Ala Ala Arg Arg
 65 70 75 80

Ala Ala Ala Arg Ser Arg Ala Ala Ala Ala Gly Gly Met Arg Arg Asp
 85 90 95

Pro Ala Pro Gly Phe Ser Met Leu Leu Phe Gly Val Ser Leu Ala Cys
 100 105 110

Tyr Ser Pro Ser Leu Lys Ser Val Gln Asp Gln Ala Tyr Lys Ala Pro
 115 120 125

Val Val Val Glu Gly Lys Val Gln Gly Leu Val Pro Ala Gly Gly Ser
 130 135 140

Ser Ser Asn Ser Thr Arg Glu Pro Pro Ala Ser Gly Arg Val Ala Leu
 145 150 155 160

Val Lys Val Leu Asp Lys Trp Pro Leu Arg Ser Gly Gly Leu Gln Arg
 165 170 175

Glu Gln Val Ile Ser Val Gly Ser Cys Val Pro Leu Glu Arg Asn Gln
 180 185 190

Arg Tyr Ile Phe Phe Leu Glu Pro Thr Glu Gln Pro Leu Val Phe Lys
 195 200 205

Thr Ala Phe Ala Pro Leu Asp Thr Asn Gly Lys Asn Leu Lys Lys Glu
 210 215 220

Val Gly Lys Ile Leu Cys Thr Asp Cys Ala Thr Arg Pro Lys Leu Lys
 225 230 235 240

Lys Met Lys Ser Gln Thr Gly Gln Val Gly Glu Lys Gln Ser Leu Lys
 245 250 255

Cys Glu Ala Ala Ala Gly Asn Pro Gln Pro Ser Tyr Arg Trp Phe Lys
 260 265 270

Asp Gly Lys Glu Leu Asn Arg Ser Arg Asp Ile Arg Ile Lys Tyr Gly
 275 280 285

Asn Gly Arg Lys Asn Ser Arg Leu Gln Phe Asn Lys Val Lys Val Glu
 290 295 300

Asp Ala Gly Glu Tyr Val Cys Glu Ala Glu Asn Ile Leu Gly Lys Asp
 305 310 315 320

Thr Val Arg Gly Arg Leu Tyr Val Asn Ser Val Ser Thr Thr Leu Ser
 325 330 335

Ser Trp Ser Gly His Ala Arg Lys Cys Asn Glu Thr Ala Lys Ser Tyr
 340 345 350

Cys Val Asn Gly Gly Val Cys Tyr Tyr Ile Glu Gly Ile Asn Gln Leu
 355 360 365

Ser Cys Lys Cys Pro Asn Gly Phe Phe Gly Gln Arg Cys Leu Glu Lys
 370 375 380

Leu Pro Leu Arg Leu Tyr Met Pro Asp Pro Lys Gln Ser Val Leu Trp
 385 390 395 400

Asp Thr Pro Gly Thr Gly Val Ser Ser Ser Gln Trp Ser Thr Ser Pro
 405 410 415

Ser Thr Leu Asp Leu Asn
 420

<210> 4
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> SYNTHESIZED SEQUENCE

<400> 4

His	Asp	Val	Glu	Thr	Gln	Phe	Asn	Gln	Tyr	Lys	Thr	Glu	Ala	Ala	Ser
1				5					10					15	

Cys

<210> 5
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> SYNTHESIZED SEQUENCE

<400> 5

His	Asp	Val	Glu	Thr	Gln	Phe	Asn	Gln	Tyr	Lys	Thr	Glu	Ala	Ala	Ser
1				5					10					15	

<210> 6
<211> 17
<212> PRT
<213> Artificial Sequence

<220>

<223> SYNTHESIZED SEQUENCE

<400> 6

Gln Tyr Lys Thr Glu Ala Ala Ser Arg Tyr Asn Leu Thr Ile Ser Asp
1 5 10 15

Cys

<210> 7

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> SYNTHESIZED SEQUENCE

<400> 7

Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro Phe Ser Ala Gln Ser
1 5 10 15

Gly Ala Cys

<210> 8

<211> 19

<212> PRT

<213> Artificial Sequence

<220>

<223> SYNTHESIZED SEQUENCE

<400> 8

Asp Val Glu Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser Arg

1

5

10

15

Tyr Asn Leu

<210> 9

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> SYNTHESIZED SEQUENCE

<400> 9

Asn Ile Lys Phe Arg Pro Gly Ser Val Val Val Gln Leu Thr Leu Ala

1

5

10

15

Phe Arg Glu Cys

20

<210> 10

<211> 1054

<212> DNA

<213> Homo sapiens

<400> 10

ccctgcagaa gccgcgctcc ctactcggtc cgggggcaga gggggcgcca gagagcaagt 60

gggcgggcgt cccatcctcc gcatacctct ccaggtcctg gcgcacaggg tgggagcgct 120

gcgctgcgcc gcgctgcgca tcgcggcccg ctgtccgcct gccccctgcc ctagctgggc 180
cacctccccg ggctgccggt ggagggctaa gaggcgctaa cgttacgctg tttccggttt 240
tccagcgggc tctgtttccc ctcccaaggc ggcgggcggct gagcggcgga gcccccaaa 300
tggcctggcc agatgcggca ggtttgctgc tcagcgcctgc cgccgccgcc actggagaag 360
ggtcggtgca gcagctacag cgacagcagc agcagcagca gcgagaggag cagcagcagc 420
agcagcagca gcagcgagag cggcagcagc agcaggagca gcagcaacaa cagcagcatc 480
tctcgtcccc ctgcgcccc agagccgcgg ccgcagcaac agccgcagcc ccgcagcccc 540
gcagcccgga gagccgccgc ccgttcgcga gccgcagccg ccggcggcat gaggcgcgac 600
ccggcccccg gcttctccat gctgctcttc ggtgtgtgc tcgcctgcta ctgcccagc 660
ctcaagtcag tgcaggacca ggcgtacaag gcacccgtgg tggaggagg caaggtacag 720
gggctgggtcc cagccggcgg ctccagctcc aacagcacc gagagccgcc cgcctcgggt 780
cgggtggcgt tggtaaaggt gctggacaag tggccgctcc ggagcggggg gctgcagcgc 840
gagcaggtga tcagcgtggg ctctgtgtg ccgctcgaaa ggaaccagcg ctacatcttt 900
ttcttgagc ccacggaaca gcccttagtc tttaagacgg cctttgcccc cctcgatacc 960
aacggcaaaa atctcaagaa agaggtgggc aagatcctgt gcactgactg cggtgagtgc 1020
ccccctccct ttgctggaga aaggggggag gggc 1054

<210> 11

<211> 419

<212> DNA

<213> Homo sapien

<400> 11

gcactgcccc taccaccacc gtgctcacct acctgccttg taaccacacag ccacccggcc 60

caagttgaag aagatgaaga gccagacggg acaggtgggt gagaagcaat cgctgaagtg 120

tgaggcagca gccggtaatc cccagccttc ctaccgttgg ttcaaggatg gcaaggagct 180

caaccgcagc cgagacattc gcatcaaata tggcaacggc aggtaagcaa tatccagcct 240

gtcctccatc caagcagctg ccctccctcc tctcgagaag gagtggcctg aactgggcag 300

aggccagggc tgtgccccca gactcaccca tggaaccagt cccagcccct ctccatactc 360

cccttccttg ccactcccag ctttcttctt gcagctcggg gaccctctgg tcatgggtgg 419

<210> 12

<211> 493

<212> DNA

<213> Homo sapien

<400> 12

gccactagtc ctgccctgtg ttgtcctaag ggcccaaggc tccctgcagc tgcagggact 60

ccgtgccccca cctggactcc agaaattgct ggggggaggg aatctccttc ccatctaagc 120

tgcagggcca ctgtggctct gtggagagag gcaaccgctg ggtgactgct ggggagccac 180

agccggccct ggctcacgcc tctccccctt ttatccccct cctaaccaga aagaactcac 240

gactacagtt caacaagggtg aagggtggagg acgctgggga gtatgtctgc gaggccgaga 300

acatcctggg gaaggacacc gtccggggcc ggctttacgt caacagcggg aggtgggccc 360

agacagaggg aagggctcta gaggggtttg gcagggcggg ccagtggccc cccagccctt 420

ggggcctgca tgtgatccat ctgcactca actccccctc ctctgtctgc tgccatatca 480

agccctagat ctc

493

<210> 13

<211> 350

<212> DNA

<213> Homo sapien

<400> 13

ggtcacatcttc cagtttttgac gtggggcatg aaggagatga ttccctggggc ctagggatag 60

tctcagtgcg tcactggcac atgtctctca taccctcagt gagcaccacc ctgtcatcct 120

ggtcgggggca cgcccgggaag tgcaacgaga cagccaagtc ctattgcgtc aatggaggcg 180

tctgctacta catcgagggc atcaaccagc tctcctgcaa gtaagtgacc agtaggggtg 240

ggcatgggag caagaacagg gtaggagatg ctgggtcaga agtggagggc tctaggaaaa 300

gaggggttcca agccactgac aagagggtccc caaggggtgt agacaggaag 350

<210> 14

<211> 326

<212> DNA

<213> Homo sapien

<400> 14

aagtgcctga cttgggttttt ctcatcctac actgtgcact ttgatttcta accttagcct 60

cgcggatgat gccagtttgt tctgtgattt ttcccttttt ccttctccag atgtccaaat 120

ggattcttcg gacagagatg ttgggagaaa ctgcctttgc gattgtacat gccagatcct 180

aagcaaagta tgtttgaaga taaaaacaca agtccctgct ccttagaaaag cttctgggtg 240

cagtcccccc aatgtagggc atagggccag ggggtgttggg tgttttgatc tttggctgtt 300

ttgtttttgtt tctcttttct tttttg 326

<210> 15

<211> 310

<212> DNA

<213> Homo sapien

<400> 15

aatacaaatg gtaacggtgg caaggaaccc agctggcatt ggcaggagcc cgtgccgggg 60

tgttgcaatg cctgtgtctt gctatcctgg ctctctcttt ctggttttct ttcttttggg 120

aggtgtcctg tgggatacac cggggacagg tgtcagcagt tcgcaatggg caacttctcc 180

agtatgtctc tttcttctat ttcttctctt ccctgggtgac tgacagtctc cccctgggga 240

gggatggggc cttgcttagc aagactaggg ggagatgtag aggggtgcct gcgctcctta 300

gaggagacgg 310

<210> 16

<211> 259

<212> DNA

<213> Homo sapien

<400> 16

ggatggaaag gagctgggca ggacctgagc tggggagagc ctatggcttt gggggcaaag 60

gggtctctgc accactatcc ctatgggggtt tgctctctgc cccacccctc acatctaccc 120

tacctctgca gaagccgagg agctgtacca gaagaggggc ctgaccatca cgggcatctg 180

cgtggctctg ctggctgtgg gcatcgtctg tgtgggtggc tactgcaaga ccaagtgagt 240

gtcactcact caagctggg 259

<210> 17
<211> 258
<212> DNA
<213> Homo sapien

<400> 17
ttgtgaccct cgctcttctg cactgccaga gcaccttgga tttgaattaa agggatatgaa 60
acctctgtgt gtcactcagc accatctgcg tggcccctct ccacagcctc cgatgccatg 120
tgtgctgaat gtttggtttc ctttccaaaa aatggaaaag ggatgattcc aaaaatctct 180
cctggcccca tcgagatgtc ctatcataaa ctacgttgat ttagcaggag acgaaagaag 240
ctcatgaaca gaagggca 258

<210> 18
<211> 8181
<212> DNA
<213> Homo sapien

<220>
<221> misc_feature
<222> (6890)..(7960)
<223> definition of "n" in this sequence is unavailable at this time

<220>
<221> misc_feature
<222> (6890)..(7960)
<223> unknown nucleotide

<400> 18
gaattcagaa ttttagaccc tttggccttg gggatccatcc tggagaccct gaggtctaag 60

ctacagcccc	tcagccaacc	acagaccctt	ctctggctcc	caaaaggagt	tcagtcccag	120
agggtgggtca	cccacccttc	agggatgaga	agttttcaag	gggtattact	caggcactaa	180
ccccaggaaa	gatgacagca	cattgccata	aagttttggt	tgttttctaa	gccagtgcaa	240
ctgcttattt	tagggatttt	ccgggatagg	gtggggaagt	ggaaggaatc	ggcgagtaga	300
agagaaagcc	tgggaggggtg	gaagttaggg	atctagggga	agtttggctg	atttggggat	360
gcgggtgggg	gaggtgctgg	atggagttaa	gtgaaggata	gggtgcctga	gggaggatgc	420
ccgaagtcct	cccagacca	cttactcacg	gtggcagcgg	cgacactcca	gtctatcaaa	480
gatccgccgg	gatggagagc	caggaggcgg	gggctgcccc	tgaggtagcg	gggaggccgg	540
ggggccgggg	ggcggacggg	acgagtgcaa	tattggcggg	ggaaaaaca	acactgcacc	600
gcgtcccgtc	cctcccggcc	gcccggggcc	ggatcccgtc	ccccaccgcc	tgaagccggc	660
ccgaccggga	accggggccg	ctggggagtt	gggttcacct	tggaggccag	agagacttgg	720
cgcccgggaag	caaagggaat	ggcaaggggg	aggggggagg	gagaacggga	gtttgcggag	780
tccagaaggc	cgctttccga	cgcccggggc	ttgcgcgcgc	ttgctcttta	agtactcaga	840
ctgcgcggcg	cgagccgtcc	gcatggtgac	gcgtgtccca	gcaaccgaac	tgaatggctg	900
ttgcttggca	atgccgggag	ttgaggtttg	gggccgcccc	cctagctact	cgtgttttct	960
ccggcctgcg	agttgggggg	ctcccgctc	cccggccccg	tcctggggcg	gctgacgtca	1020
gatgtcccca	ccccgcccag	cgcttgcccc	aagggtctcg	ccgcacacaa	agctcggcct	1080
cgggcgccgg	cgcgcggggc	agagcgggtg	tctctcgctt	gctgatctga	tgcgctccaa	1140
tcccgtgcct	cgccgaagtg	tttttaaagt	gttctttcca	acctgtgtct	ttggggctga	1200

gaactgtttt	ctgaatacag	gcggaactgc	ttccgtcggc	ctagaggcac	gctgcgactg	1260
cgggacccaa	gttcacgtg	ctgccgcggc	ctgggatagc	ttcctcccct	cgtgcactgc	1320
tgccgcacac	acctcttggc	tgtcgcgcac	tacgcacctc	acgtgtgctt	ttgccccccg	1380
ctacgtgcct	acctgtcccc	aataccactc	tgctccccaa	aggatagttc	tgtgtccgta	1440
aatcccattc	tgtcacccca	cctactctct	gcccccccct	tttttgtttt	gagacggagc	1500
tttgctctgt	cgcccaggct	ggagtgcac	ggcgcgatct	cggctcactg	caacctccgc	1560
ctcccggggt	caagcgattc	tcctgcctca	gcctcctgag	tagctggggg	tacagcgccc	1620
gccaccacgc	tcggctaatt	tttgtagttt	ttagtagaga	cgaggtttca	ccatcttggc	1680
caggctggtc	ttgaaccctc	gaccttgtga	tccactcgcc	tcggccttcc	aaagtgttgg	1740
gattacgggc	gtgacgaccg	tgccacgcac	ctgcctctta	agtacataac	ggccacaca	1800
gaacgtgtcc	aactcccccg	cccacgttcc	aacgtcctct	cccacatacc	tcggtgcccc	1860
ttccacatac	ctcaggaccc	caccgcctta	gtccatttcc	ctccagacgc	caccaccacg	1920
cgtcccggag	tgccccctcc	taaagctccc	agccgtccac	catgctgtgc	gttcctccct	1980
ccctggccac	ggcagtgacc	cttctctccc	gggcccctgt	tcctctctgc	gggctctgct	2040
gcctcactta	ggcagcgctg	cccttaactc	tctccgcccc	gtccgagcgg	cccctcagct	2100
tcggcgccca	gccccgcaag	gtccccggtg	accactagag	ggcgggagga	gtccttgccc	2160
agtgggtggg	agtggcaagg	aaggacccta	gggttcatcg	gagcccaggt	ttactccctt	2220
aagtggaaat	ttcttcccc	actcctcctt	ggctttctcc	aaggagggaa	cccaggctgc	2280
tggaaagtcc	ggctgggggg	gggactgtgg	gttcagggga	gaacgggggtg	tggaacggga	2340

cagggagcgg	ttagaagggt	ggggctattc	cgggaagtgg	tggggggagg	gagcccaaaa	2400
ctagcaccta	gtccactcat	tatccagccc	tcttatttct	cgcccgctct	gcttcagtgg	2460
acccggggag	ggcggggaag	tggagtggga	gacctagggg	tgggcttccc	gaccttgctg	2520
tacaggacct	cgacctagct	ggctttgttc	cccatcccca	cgttagtgtg	tgccctgagg	2580
ctaaaactag	agcccagggg	ccccaagttc	cagactgccc	ctccccctc	ccccggagcc	2640
agggagtggg	tggtgaaagg	gggaggccag	ctggagaaca	aacgggtagt	caggggggtg	2700
agcgattaga	gcccttgtac	cctaccagg	aatgggttgg	gaggaggagg	aagaggtagg	2760
aggtagggga	gggggcgggg	ttttgtcacc	tgtcacctgc	tcgctgtgcc	tagggcgggc	2820
gggcggggag	tggggggacc	ggtataaagc	ggtaggcgcc	tgtgcccgtc	ccacctctca	2880
agcagccagc	gcctgcctga	atctgttctg	ccccctcccc	accattttca	ccaccaccat	2940
gacaccgggc	accagtcctc	ctttcttctc	gctgctgctc	ctcacagtgc	ttacaggtga	3000
ggggcacgag	gtggggagtg	ggctgccctg	cttaggtggg	cttcgtgggc	tttctgtggg	3060
ttttgctccc	tggcagatgg	caccatgaag	ttaaggtaag	aattgcagac	agaggctgcc	3120
ctgtctgtgc	cagaaggagg	gagaggctaa	ggacaggctg	agaagagttg	cccccaacc	3180
tgagagtggg	taccaggggc	aagcaaagt	cctgtagaga	agtctagggg	gaagagagta	3240
gggagagggg	aggcttaaga	ggggaagaaa	tgcaggggcc	atgagccaag	gcctatgggc	3300
agagagaagg	aggctgctgc	agggaaggag	gcttccaacc	caggggttac	tgaggctgcc	3360
cactccccag	tcctcctggg	attatttctc	tgggtggccag	agcttatatt	ttcttcttgc	3420
tcttattttt	ccttcataaa	gaccaaaccc	tatgacttta	acttcttaca	gctaccacag	3480

cccctaaacc	cgcaacagtt	gttacagggt	ctgggtcatgc	aagctctacc	ccagggtggag	3540
aaaaggagac	ttcgggtacc	cagagaagtt	cagtgtcccag	ctctactgag	aagaatgctg	3600
tgagtatgac	cagcagcgta	ctctccagcc	acagccccgg	ttcagggtcc	tccaccactc	3660
agggacagga	tgtcactctg	gccccggcca	cggaaccagc	ttcagggtca	gctgccacct	3720
ggggacagga	tgtcacctcg	gtcccagtc	ccaggccagc	cctggggtcc	accacccccg	3780
cagccccacga	tgtcacctca	gccccggaca	acaagccagc	cccggggtcc	accgcccccc	3840
cagccccacgg	tgtcacctcg	gccccggaca	ccaggccggc	cccggggtcc	accgcccccc	3900
cagccccatgg	tgtcacctcg	gccccggaca	acaggccggc	cttggggtcc	accgccccct	3960
cagtccacaa	tgtcacctcg	gcctcaggt	ctgcatcagg	ctcagcttct	actctgggtg	4020
acaacggcac	ctctgccagg	gctaccacaa	ccccagccag	caagagcact	ccatttctca	4080
ttcccagcca	ccactctgat	actcctacca	cccttgccag	ccatagcacc	aagactgatg	4140
ccagtagcac	tcaccatagc	acgggtacct	ctctcacctc	ctccaatcac	agcacttctc	4200
cccagttgtc	tactggggtc	tctttctttt	tcctgtcttt	tcacatttca	aacctccagt	4260
ttaattcctc	tctggaagat	cccagcaccg	actactacca	agagctgcag	agagacattt	4320
ctgaaatggg	gagtatcggc	ctttccttcc	ccatgtctcc	ctgaagcagc	catcagaact	4380
gtccacaccc	tttgcacaa	gcccaggtcc	tttccctctc	accccagttt	ttgcagattt	4440
ataaacaagg	gggttttctg	ggcctctcca	atattaagtt	caggtacagt	tctgggtgtg	4500
gaccacagtgt	ggtgggttga	gggttgggtg	gtgggtcatga	ccgtaggagg	gactgggtgca	4560
cttaagggttg	ggggaagagt	gctgagccag	agctggggacc	cgtgggtgaa	gtgcccattt	4620

cctgtgacc	aggccaggat	ctgtggtggt	acaattgact	ctggccttcc	gagaaggtac	4680
catcaatgtc	cacgacgtgg	agacacagtt	caatcagtat	aaaacggaag	cagcctctcg	4740
atataacctg	acgatctcag	acgtcagcgg	tgaggctact	tccctggctg	cagccagcac	4800
catgccgggg	cccctctcct	tccagtgtct	gggtccccgc	tctttcctta	gtgctggcag	4860
cgggaggggc	gcctcctctg	ggagactgcc	ctgaccactg	cttttccttt	tagtgagtga	4920
tgtgccatth	cctttctctg	cccagtctgg	ggctgggggtg	ccaggctggg	gcatcgcgct	4980
gctggtgctg	gtctgtgttc	tggttgcgct	ggccattgtc	tatctcattg	ccttggtgag	5040
tgcagtccct	ggccctgata	agagcccccc	ggtagaaggc	actccatggc	ctgccataac	5100
ctcctatctc	cccaggctgt	ctgtcagtgc	cgccgaaaga	actacgggca	gctggacatc	5160
tttccagccc	gggataccta	ccatcctatg	agcgagtacc	ccacctacca	cacccatggg	5220
cgctatgtgc	cccctagcag	taccgatcgt	agcccctatg	agaagggtgag	attgggcccc	5280
acaggccagg	ggaagcagag	ggtttggctg	ggcaaggatt	ctgaaggggg	tacttggaag	5340
acccaaagag	cttggaagag	gtgagaagtg	gcgtgaagtg	agcagggggag	ggcctggcaa	5400
ggatgagggg	cagaggtcag	aggagtthttg	ggggacaggc	ctgggaggag	actatggaag	5460
aaaggggccc	tcaagaggga	gtggccccac	tgccagaatt	cctaaaagat	cattggccgt	5520
ccacattcat	gctggctggc	gctggctgaa	ctggtgccac	cgtggcagtt	ttgtttttgtt	5580
ttgctthttt	gcaccagag	gcaaatggg	tggagcacta	tgcccagggg	agcccttccc	5640
gaggagtccc	aggggtgagc	ctctgtgccc	ctaatactct	cctaggaatg	gagggtagac	5700
cgagaaaggc	tggcataggg	ggaggthttcc	caggtagaag	caagaagaag	tgtcagcaga	5760

ccaggtgagc	gtgggtgcc	gtggggttct	tgggagcttc	aaggaagcaa	ggaacgctcc	5820
ctccttcctc	tcctgggtctt	tctctatggg	acctagtaaa	taattactgc	agccacctga	5880
ggctggaaaa	ccactccagg	tgggggagga	gagagtttag	ttttcttgct	cctattttcc	5940
tcctcctgga	gacctccctc	tctcggtttt	acaaagacac	agatacacc	cgcccccaa	6000
aacacacaca	cacacacaca	cacacacacc	tccttaggct	ggaacagcag	agaatggagg	6060
gacaaggggg	ctgattagag	ccaagaagag	ggagtgaagg	agagcagagg	gaggagggca	6120
gccctgttta	cagtcacctg	gctgggtggg	tggcaggtgc	tctctctgaa	ttaacccttt	6180
gagagctggc	caggactctg	gactgattac	cccagcctgg	ggtggcatcc	aggggctcta	6240
ggaggtacct	tttgctcctc	accctggatc	tcttttcctt	ccaccaggt	ttctgcaggt	6300
aatggtggca	gcagcctctc	ttacacaaac	ccagcagtgg	cagccacttc	tgccaacttg	6360
taggggcacg	tcgcccgtg	agctgagtgg	ccagccagtg	ccattccact	ccactcaggt	6420
tcttcagggc	cagagcccct	gcaccctgtt	tgggctggtg	agctgggagt	tcaggtgggc	6480
tgctcacacg	tccttcagag	gccccaccaa	tttctcggac	acttctcagt	gtgtggaagc	6540
tcagtgtggc	ccctgaggct	catgcctggg	aagtgttgtg	gtgggggctc	ccaggaggac	6600
tggcccagag	agccctgaga	tagcggggat	cctgaactgg	actgaataaa	acgtggtctc	6660
ccactggcgc	caacttctga	tctttcatct	gtgaccctg	ggcagcaggg	cgtcagaatg	6720
tgtgtgaggg	ggctggggga	ggagacaggg	aggccaggag	gcagtaagga	gcgagtttgt	6780
ttgagaagca	ggagatgtga	ggaggaggtg	acattgggga	gtaggggtgg	cctgaggagc	6840
cacctctggc	taaccctggc	agcacaagag	gaaggaggaa	acgaaacca	ggcnggcttt	6900

ggagggctag cgtgactggg ctccgtgact gagctctgtg tgccagtggc tctcccctct 6960
cctcgcctgg cccacgccct ccttgcccct ggcatgggtg cccccagggtg gctctattct 7020
tagctgtccg ggtgtgaagt aaatccttgg gcagtgataa cagcccagag tcaacagggg 7080
tgagataagc agaggctggg tcagatccgg gcgctggcac caggcccagc cccctccctg 7140
accccggtcn ccccaccagc ctgctgcccc tgggggtggnc tccacaacac cctgggaatg 7200
gggaagtggg tctggttccc tgaccccttt ggcccaggca cggtgcctgt ccctcgaccg 7260
cattccccca gggcctgtgc tgcaggcctg gaagccctga ttggggcctg ccaccagcag 7320
ccagagagct atgttccttg gcagctgtga tgcgctcagg ccgggccagg acacgtgtgg 7380
caggaggctt agagcacctg cctggggcct tcctctctca ggcaccagat ccattggttg 7440
ctcctgccta gaaccacagc ctagcacccc tgctccctcc cgcctaccac acccagcaca 7500
gaaactcaca ggaatgattg cgctcagggg aggcagagat gtgcctggca tcacagttta 7560
ttgtttataa accatgacaa taacagctgt tgctcagcac aggcctagca gagccactg 7620
cagggggacg gcagcgggca ccagaggcct tgcttggccc aacccaatgg gaacaccag 7680
actcagctgg gtccccaagg gagacttggc acattggcat ggggtgtggg caggtaaagc 7740
atgcaagagg gagaagaggg acataagggg catgcggctg cgggggtgtt ggacccaaat 7800
aaataaagca ggatgacagg gtccccttcc cctcaccagg aatgcctgac agcgtccagc 7860
cccaaagcct gcctgtccca aggctgtagt tcagcatcaa cagggcaggg agcttggcag 7920
ggcaagggca gagctggaga tcatgcccag tnttcagggt gccctccctc ccaatcagcc 7980
tggggggcac aggacaggga tggagaaggg gctctctcca tggcttgggt aacatgccaa 8040

aggcaggtca tagggcagac tcagtggggg tgggggcctg gctaacaagc aatggagaga 8100

acggggggcca tccagagagg ttggcagaag agagcccctg ggtcaagaga aaactttggg 8160

gaagacaaga cacgggagaa g 8181